THE ROLE OF INTRACELLULAR pH AND CALCIUM IN THE REGULATION OF CELLULAR FUNCTIONS

BY

Raul Martínez-Zaguilán

A Dissertation Submitted to the Faculty of the DEPARTMENT OF BIOCHEMISTRY In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA

1992
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The role of intracellular pH and calcium in the regulation of cellular functions

Martínez-Zaguilán, Raul, Ph.D.
The University of Arizona, 1992
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A Dissertation Submitted to the Faculty of the DEPARTMENT OF BIOCHEMISTRY In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 1992
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Raul Martinez-Zaquilan entitled The Role of Intracellular pH and Calcium in the Regulation of Cellular Functions and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director
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SIGNED: __________________________
ACKNOWLEDGMENTS

This thesis is dedicated to the memory of my parents, but most especially to my mother, Eufrocina Zaguián. Her love, care and confidence in me have made my success in life possible. To my brother, Demetrio who has always been there when I needed. To my other brothers and sisters (José Luis, César, Paty, Luli, Rosy, Yoli, Josan, Eli, and finally to my five years old sister, Tatiana). They have always inspired me to performed better.

Special thanks to my loving and caring wife, Gloria. Together we have spent many hours of work at the lab. She has worked side by side with me, for several years and has never complained or regretted. We have experienced together the excitement behind many of the key experiments presented in this thesis. She also has criticized many of the experiments performed and has inspired me to do better. I'm so proud to have her as my best friend, collaborator, and wife.

I express my gratitude to my advisor, Dr. Robert Gillies. He gave me the opportunity to work in his lab during the summer 1986. Since then, it has been always fun and always exciting to work for Bob. He also trusted me and allowed me to move on my own way in the lab, taking good care that I didn't disperse myself in a sea of confusion, focusing experiments without losing track of the "big picture". Bob has been an instrumental part in my ability to develop interactions with other people. I could not ask for more than to have in the same person my advisor, my professor, and my best friend.

I thank to Dr. Alejandro Reyes, who introduce me to the joy of science in my earlier years, during the summer of 1980. I thank Ken Giuliano for all the help, advice and friendship that he provide me when I started working in Bob's lab. I also thank to Pat Hoyer for having me as one of her collaborators in the field of reproduction.

Most of the scientific work involves the collaboration and/or contribution from multiple sources. This thesis is not the exception. Therefore, I would like to acknowledge the contribution of many people with whom I have the opportunity and pleasure to interact. The contribution of those individuals that has resulted in a scientific paper either published, submitted for review or in preparation, is properly acknowledge at the beginning and at the end of each section.

Special thanks to the members of my advisor committee: Neil MacKenzie, Steve Wright, Gordon Tollin, Pat Hoyer and Robert Gillies.

Finally thanks to all my friends and colleagues that have always trusted me.
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SECTION 4 DIFFERING EFFECTS OF LAC$_3$ ON CYTOSOLIC FREE CALCIUM IN OVINE LARGE AND SMALL LUTEAL CELLS.

ABBREVIATIONS USSED.
ABSTRACT

Upon cell stimulation with hormones and other mitogens, a variety of biochemical and physiological responses occur within the first few minutes, including turnover of inositol phospholipids, activation of protein kinase C and tyrosine kinases, and changes in intracellular pH (pH\text{in}) and calcium ([Ca\textsuperscript{2+}]\text{in}). Changes in both pH\text{in} and [Ca\textsuperscript{2+}]\text{in} are prominent and play a major role in the signal transduction mechanism leading to the physiological response, i.e. secretion, neurotransmission, proliferation and differentiation. The intracellular pH changes that follow mitogenic activation are complex and may reflect several different H\textsuperscript{+} transporting mechanisms. There are at least three main systems involved in the regulation of pH\text{in} in eukaryotic cells: (a) the mitogen stimulated Na\textsuperscript{+}/H\textsuperscript{+} exchange, which electroneutrally raises pH\text{in} and can be inhibited by amiloride and its derivatives; (b) a variety of HCO\textsubscript{3}\textsuperscript{-}-based mechanisms which can alkalize or acidify the cytosol, and can be inhibited by stilbene disulfonate derivatives; (c) and a plasma membrane H\textsuperscript{+}-ATPase, which represents the least understood mechanism of pH\text{in} regulation. Under non-pathological conditions, pH\text{in} regulation is generally achieved by Na\textsuperscript{+}/H\textsuperscript{+} exchange and HCO\textsubscript{3}\textsuperscript{-}-based mechanisms. Misexpression of a H\textsuperscript{+}-ATPase in the plasma membrane can lead to a chronically high pH\text{in} in some tumor cells and might contribute to carcinogenesis. Chapter I explains the dissertation format that I have chosen and
explain the relationship of the manuscripts that I have included in three appendices. This chapter also indicates my contribution to each of these manuscripts. Chapter II is a summary of the most important findings in these manuscripts. Appendix I deals with the role of Na⁺/H⁺ exchange and Cl⁻/HCO₃⁻ exchange in the regulation of pHᵢⁿ. Appendix II deals with the role of H⁺-ATPase in the maintenance of a chronically high pHᵢⁿ and its possible involvement in tumorigenesis. Although changes in pHᵢⁿ and [Ca²⁺]ᵢⁿ have been observed in many systems, it is not yet clear whether these ionic events work independently in the activation pathway leading to a particular physiological response. Appendix III looks at this problem and describes a technique to simultaneously measure pHᵢⁿ and [Ca²⁺]ᵢⁿ by fluorescence spectroscopy. This appendix also describes the application of this technique to study the role of pHᵢⁿ and Ca²⁺ in the regulation of cell growth and progesterone secretion.
CHAPTER 1.

INTRODUCTION
This dissertation prospectus represents a compilation of the experimental work that I have been performing under the advise of Dr. Gillies, first at Colorado State University where I started the Ph.D. program (three manuscripts), and then at this University (eight manuscripts). From this total of eleven manuscripts, eight are already published; two are submitted for publication; and one is in preparation. My contribution to each manuscript in terms of the generation of ideas and the performance of experiments is indicated in parenthesis as % effort, and it should only be consider as a qualitative approximation of my involvement in each manuscript. In this dissertation, the first author correspond to the person that wrote the manuscript.

For the purpose of presentation, I divided this work in three main appendices. Each appendix is further subdivided in sections that represent a manuscript either published, submitted for publication, or in preparation (vide supra).

Appendix I deals with the interrelationship between two of the most widely accepted mechanisms of pH\textsuperscript{in} regulation in mammalian cells, namely Na\textsuperscript{+}/H\textsuperscript{+} exchange and HCO\textsubscript{3}/Cl\textsuperscript{-} exchange, and its relationship to cell proliferation. In section 1 of this appendix, we demonstrate that serum deprivation causes changes in the sensitivity of 3T3 cells to further stimulation of Na\textsuperscript{+}/H\textsuperscript{+} exchange by serum (80\% effort). It has been largely hypothesized that the activity of Na\textsuperscript{+}/H\textsuperscript{+} exchanger is regulated by...
phosphorylation of the exchanger by kinase C. Our findings in section 2 indicate that in some cells this is the case (i.e. human fibroblasts), but not in others (3T3 cells). Furthermore, inhibition of kinase C does not impair the activation of Na\(^+\)/H\(^+\) exchange by serum. Therefore, we conclude that phosphorylation of the exchanger by kinase C is not the sole signal required for the activation of this exchanger (60% effort). In section 3, our data suggest that in some cell types, cell differentiation may play a role in the regulation of Na\(^+\)/H\(^+\) exchange since treatment of 3T3 cells with dimethylsulfoxide (DMSO), a well known differentiating agent, renders these cells sensitive to kinase C activators (80% effort). These earlier experiments predicted that, under *in vitro* culture conditions, i.e. pH 7.4 and presence of HCO\(_3^−\), there would be no pH change upon serum or growth factor stimulation. We have further explored this possibility in section 4 and have found that indeed, there is no pH change under *in vitro* culture conditions however, under *in vivo* conditions (i.e. pH 7.0-7.1 and presence of physiological HCO\(_3^−\)), there is a modest pH change upon serum stimulation (80% effort).

In an attempt to further clarify whether or not pH changes are needed for cell growth and/or tumorigenesis to occur, we explore a third, less understood mechanism of pH\(^a\) regulation, i.e. H\(^+\)-ATPases. Our results from these findings are expressed in Appendix II. These experiments were initiated by the finding of Perona and Serrano, that gene transfection of NIH 3T3 cells with the yeast H\(^+\)-ATPase renders
them tumorigenic (Nature, 334: 438, 1988). One of the consequences of the transfection of a H⁺-ATPase in the plasma membrane of 3T3 cells is the maintenance of a high intracellular pH, a high rate of H⁺ extrusion and a high rate of glycolysis, as expressed in section 1 of this appendix (50% effort). We have further asked whether or not a plasma membrane H⁺-ATPase might be involved in tumorigenicity by screening for the presence of this H⁺-ATPase in other rodent tumor and non tumor cells, using non-specific inhibitors of H⁺-ATPase such as N-ethylmaleimide, suramin and fusidic acid. The results of this preliminary screening were disappointing due to the non-specificity of these inhibitors and probably, also to the selection of cell types that were not the most appropriate. These results are found in section 2 (60% effort). In an attempt to correct for this mistake, we have screened for the presence of H⁺-ATPase in primary human tumor and non tumor cells, by using better inhibitors, such as the P type H⁺-ATPase inhibitor (SCH28080), and the V-type H⁺-ATPase inhibitor (bafilomycin). The results of these findings are described in section 3 (60% effort). Briefly, by using this approach we have found that five out of fourteen human tumor cells seems to have a bafilomycin sensitive H⁺-ATPase. Through simultaneous measurement of endosomal/lysosomal pH (pH\text{sec}) and cytoplasmic pH (pH\text{cyt}) by fluorescence spectroscopy, we have observed that the effect of bafilomycin can occur at either the endosome-lysosome membrane and/or at the plasma membrane. Interactions with any of these membranes would result in a decrease in pH\text{cyt}. Re-screening of human tumor cells for bafilomycin-sensitivity
and by measuring pH^{cyt} and pH^{ac} simultaneously have indicated that, one out of five human tumor cells clearly exhibit a bafilomycin sensitive component at the plasma membrane level, which suggests that misexpression of a V-type H^{+}-ATPase at the plasma membrane might occur physiologically and might be relevant to carcinogenesis.

In an attempt to further understand the relationship between pH^{in} and [Ca^{2+}]^{in}, we developed a technique that allows us to measure simultaneous changes in these two ions. The results of these efforts are expressed in Appendix III. In section 1 of this appendix, the development and application of this technique to simultaneously measure pH^{in} and [Ca^{2+}]^{in} by using fluorescence spectroscopy are fully described (70% effort). From Appendix I we learned that kinase C was not the sole regulator of Na^{+}/H^{+} exchanger. Our data in section 1 of Appendix III, indicate that Na^{+}/H^{+} exchange can be regulated by Ca^{2+}, possibly through activation of that calmodulin kinase. We have further applied this technique to understand the role of [Ca^{2+}]^{in} and pH^{in} in the regulation of luteolysis in ovine corpora lutea, as a result of our collaborative work with Dr. Pat Hoyer (Dept. Physiology). These studies indicated that [Ca^{2+}]^{in} plays a major role in the regulation of progesterone secretion in large luteal cells. The mechanisms of Ca^{2+} regulation are distinct between small and large luteal cells. Large cells are exquisitely sensitive to PGF_{2\alpha}, a major luteolytic signal in the ovine ovary. In contrast, small cells do not increase [Ca^{2+}]^{in} upon stimulation with
this hormone. Furthermore, using kinetic analysis of the PGF$_{2a}$-induced Ca$^{2+}$ transients we were able to identify two distinct Ca$^{2+}$ pools, responsible for such transients. We are currently investigating the nature and regulation of these compartments. The results of this collaborative effort are expressed in section 2 (20% effort), section 3 (20% effort), and section 4 (20% effort). It is worthwhile to mention that my contribution to the work expressed in sections 2, 3, and 4 of appendix III has been discrete, i.e. applying my knowledge of pH$_{in}$ and [Ca$^{2+}$]$_{in}$ regulation in 3T3 cells to the luteolysis problem; and performing some of the experiments involving the measurements of these ions; is most justified to say that for the most part, these experiments were performed by Dr. Hoyer's former grad student, Dr. Julie Wegner.
CHAPTER 2.

PRESENT STUDY
The methods, results and conclusions of this study are presented in the papers appended in this dissertation. The following is a summary of the most important findings in these papers.

SUMMARY

During the development of the experimental work presented in this dissertation, I have been challenged a number of times, attempting to understand the role of pH\textsuperscript{in} and [Ca\textsuperscript{2+}]\textsuperscript{in} in the regulation of cellular functions such as cell proliferation, cell tumorigenicity, and progesterone secretion. My involvement on the studies of pH\textsuperscript{in} and [Ca\textsuperscript{2+}]\textsuperscript{in} in the regulation of cell proliferation started during the summer of 1986 with Bob Gillies, whereas my involvement in the role of pH\textsuperscript{in} and [Ca\textsuperscript{2+}]\textsuperscript{in} on progesterone secretion started as a collaborative effort with Pat Hoyer, in the fall of 1988. During most of this time, I have been trying to understand how pH\textsuperscript{in} is regulated and the relevance of pH\textsuperscript{in} changes, if any, to the control of cell proliferation and cell tumorigenicity. When I started working in this field, we didn't know if pH was a signal for cell proliferation to ensue. At the present time we still do not have a definitive answer to this question, however, I feel that now we understand a little more about the mechanisms that regulate pH\textsuperscript{in} in mammalian cells.
Since I do not want to repeat the conclusions already mentioned in each of the sections (vide supra), here I would like to recapitulate some of the main ideas and aspects behind the experimental work that have lead us to our present state of knowledge in this field.

In our earlier studies we noticed that the magnitude of the pH\textsuperscript{in} change upon serum stimulation to either acutely or chronically-serum deprived cells, in the presence of HCO\textsubscript{3}\textsuperscript{-}, resulted in slight changes in pH\textsuperscript{in}. Therefore, we concluded at that time that pH changes had only a permissive role on cell proliferation (1, 2). Many researches had previously observed larger pH\textsuperscript{in} changes upon serum addition to serum-deprived cells, therefore our earlier observations that in the presence of HCO\textsubscript{3}\textsuperscript{-} there was only a small pH\textsuperscript{in} change raised criticisms on the relevance of pH\textsuperscript{in} changes in the transition from G\textsubscript{o} to G\textsubscript{1} of the cell cycle, especially if one considers that cells normally grow in the presence of HCO\textsubscript{3}\textsuperscript{-} (vide supra). One of the mechanisms that regulates pH\textsuperscript{in} that has been widely studied is Na\textsuperscript{+}/H\textsuperscript{+} exchanger (for a review see 3, 4). It has been generally acknowledged that kinase C plays a key role in the regulation of this exchanger. Through our experiments in this regard we learned that the activity of Na\textsuperscript{+}/H\textsuperscript{+} exchange is not solely regulated by phosphorylation mediated by kinase C, but that the regulation of this exchanger is also linked to Ca\textsuperscript{2+} changes, possibly through CaM-kinase, thus emphasizing the need of further studies to understand the role of Ca\textsuperscript{2+} in the regulation of pH\textsuperscript{in} (see 5, and references therein).
Cell differentiation can also play a role in the regulation of the activity of Na\(^+\)/H\(^+\) exchanger, since cells that are not responsive to stimulation of Na\(^+\)/H\(^+\) exchanger by kinase C activators, can be rendered sensitive upon treatment with cell-differentiating agents (see 6, and references therein). From our earlier experiments it was obvious that we needed a better understanding of the role of HCO\(_3\)\(^-\) in the regulation of pH\(^{in}\) and its interrelationship with the Na\(^+\)/H\(^+\) exchanger. From these experiments we learned that, under normal culture conditions, stimulation of a quiescent cell with serum or growth factors does not result in changes in pH\(^{in}\). Furthermore, in the absence of HCO\(_3\)\(^-\), when the activity of Cl/HCO\(_3\)\(^-\) is absent, stimulation of quiescent cells with serum would result in large changes in pH\(^{in}\). At intermediate pH\(^{ex}\) (7.1-7.2), similar to that found in vivo, the pH\(^{in}\) changes did occur, but were modest (see 7, and references therein). These observations predicted that under cell culture conditions pH changes can not be considered as a signal for cell proliferation. Furthermore, these data also predict that under in vivo conditions, changes in pH\(^{in}\) might be important. At that point, we were satisfied with our understanding on the role of pH in cell proliferation, since we could safely state that pH changes played only a permissive role in cell proliferation.

Our data up to date seems to indicates that the maintenance of a "critical window" of pH\(^{in}\) is required for cell proliferation. Maintenance of pH\(^{in}\), above that predicted from Nernst equilibrium, is mediated by the activity of one, two, or three
mechanisms, including Na\(^+\)/H\(^+\) exchange, HCO\(_3^-\)-based transport mechanisms, and H\(^+\)-ATPase (for a review, see 8). In many normal cells, maintenance of pH\(^{in}\) is achieved by the first two mechanisms. In some specialized tissue, a H\(^+\)-ATPase is also involved. But, what happens under pathological conditions, such as cancer? What if we maintain a chronically high pH\(^{in}\), bypassing any of the mechanisms regulating the activity of Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3^-\) transports? Our most recent data indicates that maintenance of a chronically high pH\(^{in}\) leads to loss of growth control and tumorigenicity, even under physiological HCO\(_3^-\) concentrations. This has been clearly demonstrated in NIH-3T3 cells transfected with a gene for the H\(^+\)-ATPase. Expression of this H\(^+\)-ATPase in their plasma membrane renders these cells tumorigenic (9). These transfected cells maintain a high pH\(^{in}\) and a high rate of glycolysis (10). In human tumor cells, we believe that a V-type H\(^+\)-ATPase, which is normally present in endosomes/lysosomes and other intracellular organelles, is overexpressed in the plasma membrane and contributes to the maintenance of a high pH\(^{in}\) in these cells (cf 8, 11). This finding raises the possibility that a V-type H\(^+\)-ATPase may behave as an oncogene, since this H\(^+\)-pump is normally present in intracellular organelles where it contributes to the maintenance of the acidic environment of endosomes and lysosomes. However, when overexpressed and/or targeted to the "wrong" place, i.e. plasma membrane, it leads to a chronically high pH\(^{in}\), thereby behaves as an "oncogene". This is further supported by our observation that the sole presence of a H\(^+\)-ATPase in the plasma membrane can render a cell
tumorigenic (cf 9, 10).

Our understanding on the role of Ca\(^{2+}\) in the regulation of cellular functions, and its interrelationship to pH\(^{\text{in}}\) is still unclear, possibly due in part to the complications in measuring [Ca\(^{2+}\)]\(^{\text{in}}\) (see 12, 13 and references therein). The development of a novel technique to monitor simultaneously intracellular pH and Ca\(^{2+}\) have increased our understanding on the relationship between pH\(^{\text{in}}\) and [Ca\(^{2+}\)]\(^{\text{in}}\) in the regulation of cell growth, and progesterone secretion in ovarian cells (12, 13, 14, 15). Our studies on the role of [Ca\(^{2+}\)]\(^{\text{in}}\) in the regulation of progesterone secretion have indicated that large, but not small ovarian cells, respond with a transient increase in [Ca\(^{2+}\)]\(^{\text{in}}\) with no changes in pH\(^{\text{in}}\) in response to PGF\(_{2\alpha}\). Furthermore, kinetic analysis of these transients allowed us to suggest that there are at least two compartments involved in the regulation of these transients, i.e. a fast compartment with a low capacity, and a slow compartment with a large capacity (see ref 14, 15 and references therein). Further studies are needed to identify the nature and the regulation of these compartments.

Predicting what the future research is going to be is always a difficult task, however at this moment it is possible for me to foresee my immediate future. It is clear from the enclosed data that at the present time, we have only circumstantial evidence for the presence of a V-type H\(^{+}\)-ATPase in the plasma membrane of primary human
tumor cells. Therefore, in order to obtain an unequivocal answer to the presence of a H\textsuperscript{+}-ATPase in the plasma membrane of those cells, the following critical experiments are needed: (a) to select one primary human tumor cell that express this bafilomycin-sensitive component (e.g. mesothelioma); (b) obtain plasma membrane from these cells; (c) isolate the H\textsuperscript{+}-ATPase from the plasma membrane; (d) obtain antibodies to this enzyme to do immunocytochemistry (fluorescence and electro-immunocytochemistry); reconstitute the enzyme into liposomes; (e) evaluate sensitivity to bafilomycin. This is an ambitious proposal that could take several years before we can make a clear statement of the presence of a V-type H\textsuperscript{+}-ATPase in the plasma membrane of human tumor cells. Regarding future investigations in the area of progesterone secretion, although might seem too speculative for me to discuss, I would like to suggest several critical directions: (a) studies on the mechanisms that regulate pH\textsuperscript{in} are lacking in these cells, and our preliminary experiments indicate a role for pH in the regulation of progesterone secretion; (b) our preliminary experiments indicated that large cells possess a higher pH\textsuperscript{in} than small ovarian cells, thus raising the possibility of the presence of H\textsuperscript{+}-ATPases in these cells; (c) the development of methods to culture ovarian luteal cells in a continuous fashion, e.g. immortalizing ovarian cells by gene transfection such as c-myc, will avoid the limitations of the \textit{in vivo} experimental model, and should enhance enormously our experimental capabilities towards the understanding of the regulation of luteal functions; (d) a better understanding of the interrelationship between pH\textsuperscript{in} and
[Ca$^{2+}]_{in}$ should enhance our knowledge on the role of these two ions in the regulation of secretory processes.
REFERENCES


APPENDIX I.

REGULATION OF Na⁺/H⁺ EXCHANGE AND NaHCO₃/HCl EXCHANGE AND ITS ROLE IN MAMMALIAN CELL PROLIFERATION.
SECTION 1.

Effect of serum on the intracellular pH of BALB/c-3T3 cells: serum deprivation causes changes in the sensitivity of cells to serum.

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April 29, 1992

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Effect of Serum on the Intracellular pH of BALB/c-3T3 Cells: Serum Deprivation Causes Changes in Sensitivity of Cells to Serum

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One of the earliest responses of quiescent mammalian cells to the addition of serum is an increase in intracellular pH (pHm). This pHm change is generally believed to be due to an increased activity of Na⁺/H⁺ exchange. A number of investigators have observed steady-state differences in pHm between cells in the presence and absence of serum. However, no one has examined differences in pHm regulation that may exist between cells chronically exposed to, or deprived of serum. In this study, we investigated the effects of serum deprivation to identify those components of pHm regulation that were associated with quiescence. To do this, we examined pHm in cells growing chronically in 10% serum as well as in cells that were either acutely (1.5–2 hr) or chronically (48 hr) deprived of serum. Intracellular pH was monitored using the fluorescence of intracellularly loaded pyranine dye. Our results indicate that the resting pHm values of chronically or acutely serum-deprived cells were not significantly different from each other yet, in both cases, were lower than those observed in cells exposed to 10% serum. Furthermore, we observed significant increases in pHm of both acutely or chronically serum-deprived cells in response to the addition of serum at various concentrations, in the presence of 24 mM bicarbonate. Chronically serum-deprived cells had slightly smaller responses and were more sensitive to lower concentrations of serum than were acutely deprived cells. Therefore, our data suggest that long-term serum deprivation affects the magnitude and sensitivity of pHm to serum stimulation and causes the loss of some form of pHm regulatory mechanisms.

Most mammalian cells in culture require serum for sustained proliferation (Baserga, 1985). In the absence of serum, BALB/c-3T3 cells become totally quiescent within 48 hours (e.g., Gillies et al., 1986). One of the earliest events to occur upon the readdition of serum to serum-deprived cells is an increase in the activity of Na⁺/H⁺ exchange. A number of investigators have observed steady-state differences in pHm between cells in the presence and absence of serum. Under conditions where the cytosol is relatively acidic, this increased activity will lead to an increase in intracellular pH (pHm). A number of workers have observed steady-state differences between the pHm of cells in the presence and absence of serum (Shuldiner and Rozengurt, 1982; Cassel et al., 1983; L'Allemain et al., 1984; Poulsen et al., 1984; Hesketh et al., 1985; Bright et al., 1987). However, none of these studies has examined any differences that may exist between acutely and chronically deprived cell populations under identical conditions. These studies therefore have not addressed the question whether there are any changes in the mechanism(s) of pHm regulation that occur to cells as a consequence of serum deprivation. In the present study we address this question by comparing the pHm responses of serum-stimulated cells, cells that are deprived of serum for 48 hours ("chronically deprived"), and the intermediate case of cells that have been deprived of serum for 1.5–2 hours ("acutely deprived").

We recently investigated the effects of serum on pHm using flow cytometric analysis of BALB/c-3T3 cells loaded with the pH-sensitive fluorophore, dicyanohydroquinone (Gillies et al., 1987). In that study, we observed that there were differences in the timing and magnitude of the pHm response between BALB/c-3T3 cells that were chronically deprived and those that were only deprived of serum for only 15 minutes. Two problems in this former study were the poor time resolution (30 sec per datum) and the fact that these anchorage-dependent cells were suspended in solution. In this communication, we examine this phenomenon with much higher temporal resolution and for longer times using the fluorescence of pyranine dye loaded into BALB/c-3T3 cells attached to coverslips (Giuliano and Gillies, 1987). The results of the present study indicate that the resting pHm of cells in the absence of serum is consistently lower than that of cells in the presence of 10% serum and that there is no significant difference between the resting pHm of cells that are either chronically or acutely deprived of serum. Differences do exist between acutely and chronically serum-deprived cells.

Received September 8, 1987; accepted February 15, 1988.

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deprived BALB/c-3T3 cells, in terms of the sensitivity and the magnitude of cytoplasmic alkalinization that occurs in response to serum addition. Furthermore, the present study indicates that although physiological levels of serum do not (Giuliano and Gillies, 1987). The ability of the scrape loaded cells to regulate pH^m has been studied by Giuliano and Gillies (1987), through perfusing BALB/c-3T3 cells with CSB containing ammonium chloride to induce a cytosolic alkalinization, followed by removal of ammonium chloride to induce an acidification in the same experiment. Under these experimental conditions, the pH^m of these cells reacidified to near control levels during continuous treatment with 10 mM ammonium chloride. These cells realkalinized again to near-control levels, after an acute acid load caused by rapid removal of ammonium chloride. Therefore, these scrape-deprived cells were able to re-establish normal ion gradients and membrane permeabilities for a review, see Roos and Boron, 1984.

**pH determinations**

Intracellular pH was determined by fluorescence of intracellular pyranine (Giuliano and Gillies, 1987). Fluorescence was measured in BALB/c-3T3 cells that have been either chronically or acutely deprived of serum. This is demonstrated in Figures 1 and 2, where the pH^m of either chronically deprived (Fig. 1) or acutely deprived (Fig. 2) cells is shown in response to changing serum concentrations from 0.2% to 1% (w/v). As illustrated in these figures, chronically deprived cells respond to the addition of serum by increasing their pH^m by approximately 0.05 pH units. The pH^m of acutely deprived cells, on the other hand, does not respond to the addition of 1% serum and, in fact, slightly decreases after serum concentration increases. Figures 1 and 2 also illustrate that the resting pH^m is similar in both cell preparations. Data from a number of experiments similar to those shown in Figures 1 and 2 are summarized in Figure 3, where results are expressed as the pH^m change induced by the addition of different serum concentrations. These data show that chronically deprived cells respond to the addition of low concentrations of serum with significant increases in pH^m, whereas the pH^m of acutely deprived cells does not respond until serum concentrations are raised to 0.2%.

As shown in Figure 4, cells chronically exposed to 10% Nu-serum respond to the change of serum from 10% to 0.2% with a monotonic decrease in pH^m. We have performed this experiment a number of times and have determined that it takes 1.5-2 hr for the newly deprived cells to reach a new resting pH^m. The new resting pH^m value reached after the decrease of serum is similar to the resting pH^m determined in either acutely or chronically serum-deprived cells.

**MATERIALS AND METHODS**

Cell culture

BALB/c-3T3 mouse embryonic fibroblasts were obtained from American Type Culture Collection (ATCC CCL 63). They were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY), supplemented with 10% Nu-serum (Collaborative Research, Beverly, MA). These cells were cultured as described in Giuliano and Gillies (1987). For experiments, cells were inoculated into 90-mm petri dishes (Corning) at a density of 10^6 cells/dish in DMEM plus 10% Nu-serum. On the following day, the growth medium was changed to DMEM containing 0.2% Nu-serum (chronically serum-deprived cells), or the cultures were scrape-loaded with pyranine dye (Molecular Probes, Inc.) and subsequently handled with either 10% Nu-serum (serum-stimulated cells) or 0.2% Nu-serum (acutely serum-deprived cells). The chronically serum-deprived cells were incubated under 0.2% Nu-serum for an additional 48 hr, at which time they were scrape-loaded with pyranine and prepared for fluorometry in the presence of 0.2% Nu-serum. Previous studies have shown that after 48 hr in the presence of 0.2% serum, cultures are fully quiescent, as monitored by cell number, thymidine incorporation, and labeled nuclei (Gillies et al., 1989).

Scrape-loading of pyranine dye

Pyranine was loaded into the cells using the scrape-loading method described by McNeil et al. (1984). Briefly, the cells to be loaded were washed three times with cell suspension buffer (CSB) containing 1.3 mM CaCl_2, 1 mM MgSO_4, 5.4 mM KCl, 0.44 mM KH_2PO_4, 110 mM NaCl, 0.35 mM Na_2HPO_4, 24 mM NaHCO_3, 5 mM glucose, 2 mM glutamine, and 25 mM HEPES at a pH of 7.15 at 37°C. The cells were then mechanically harvested using a rubber policeman in the presence of 1 mM pyranine in CSB at 37°C. Suspended cells were then washed twice with ice-cold DMEM plus the same concentration of Nu-serum in which the cells were cultured. The final cell pellet was then resuspended in cell suspension buffer with cell suspension buffer

Sample fluorescence was determined using an SLM-4800 spectrophuorometer outfitted with a flow-through device for sample perfusion (Ohkuma and Poole, 1978). Attached cells were continuously perfused at 1.0 ml/min with the appropriate buffer solution as indicated in the text. Sample temperature was maintained by keeping both the cuvette’s water jacket and the perfusion media at 37°C. The complete technique used here is described elsewhere (Giuliano and Gillies, 1987).

**RESULTS**

BALB/c-3T3 cells that have been either chronically or acutely deprived of serum differ in their sensitivity to serum. This is demonstrated in Figures 1 and 2, where the pH^m of either chronically deprived (Fig. 1) or acutely deprived (Fig. 2) cells is shown in response to changing serum concentrations from 0.2% to 1% (w/v). As illustrated in these figures, chronically deprived cells respond to the addition of serum by increasing their pH^m by approximately 0.05 pH units. The pH^m of acutely deprived cells, on the other hand, does not respond to the addition of 1% serum and, in fact, slightly decreases after serum concentration increases. Figures 1 and 2 also illustrate that the resting pH^m is similar in both cell preparations. Data from a number of experiments similar to those shown in Figures 1 and 2 are summarized in Figure 3, where results are expressed as the pH^m change induced by the addition of different serum concentrations. These data show that chronically deprived cells respond to the addition of low concentrations of serum with significant increases in pH^m, whereas the pH^m of acutely deprived cells does not respond until serum concentrations are raised to 0.2%.

As shown in Figure 4, cells chronically exposed to 10% Nu-serum respond to the change of serum from 10% to 0.2% with a monotonic decrease in pH^m. We have performed this experiment a number of times and have determined that it takes 1.5-2 hr for the newly deprived cells to reach a new resting pH^m. The new resting pH^m value reached after the decrease of serum is similar to the resting pH^m determined in either acutely or chronically serum-deprived cells.
The average resting pH\textsubscript{m} values for BALB/c-3T3 cells under various conditions in our study are given in Table 1. These data were obtained by taking the average of all determinations at various pH\textsuperscript{m} values for cells that were chronically serum deprived, acutely serum deprived, or continuously exposed to 10% serum. As indicated in this table, there are no significant differences among the average pH\textsuperscript{m} employed for the three conditions. There is, however, a significant difference between the pH\textsuperscript{m} of cells in the presence of serum and the pH\textsuperscript{m} values of cells in the absence of serum for more than 1.5 hr.

The data in Table 1 indicate that there are steady-state pH\textsuperscript{m} differences between cells in the presence or absence of serum that are independent of pH\textsuperscript{m}. However, both the absolute pH\textsuperscript{m} and the magnitude of the pH\textsuperscript{m} response of chronically deprived cells are very dependent of the pH\textsuperscript{m}. In cells chronically exposed to 10% serum, however, pH\textsuperscript{m} was observed to be much less dependent on pH\textsuperscript{m}. Examples of these observations are presented in Figure 5, where pH\textsuperscript{m} values are expressed as a function of pH\textsuperscript{m} for cells chronically exposed to 10% serum (circles) and cells deprived of serum for 48 hours (triangles). In this figure, the calculated linear slopes of these data are represented by a dashed line for cells in 10% serum and a solid line for chronically deprived cells in 0.2% serum. If cells have no ability to regulate pH\textsuperscript{m} in the face of varying pH\textsuperscript{m}, we expect that the slope of the pH\textsuperscript{m} versus pH\textsuperscript{m} curve and the linear correlation coefficient for this relationship will both approach unity. On the other hand, if cells are fully able to maintain a constant pH\textsuperscript{m} at various pH\textsuperscript{m} values, the slope of this relationship will approach 0. The linear correlation coefficient in this case will decrease because we expect that the relationship between pH\textsuperscript{m} and pH\textsuperscript{m} will be bimodal (cf. Gillies et al., 1982). Intermediate cases of pH\textsuperscript{m} regulation will have slopes and correlation coefficients intermediate between these extremes. According to figure 5, the steep slope in the chronically serum-deprived case,
associated with a relatively large correlation coefficient, suggests that these cells have little ability to regulate pH in the face of varying pH conditions. On the other hand, the flatter slope and the lower correlation coefficient in the serum-stimulated case suggests that acutely deprived cells are similar to chronically serum-deprived cells in their ability to respond to serum.

The results of the above experiments are summarized in Table 2, which also includes data on the response time of pH to serum addition. These results indicate that acutely deprived cells are similar to chronically deprived cells in their ability to respond to serum.

**DISCUSSION**

In a previous study, large differences in pH were observed between chronically and acutely deprived BALB/c-3T3 cells (Gillies et al., 1987). In that study, we determined that cells deprived of serum for 96 hr have a basal pH that is lower than that of cells deprived of serum for 20 min. Addition of serum to either these chronically or acutely deprived cells resulted in a cytosolic alkalinization, with the chronically deprived cells responding more slowly than the acutely deprived cells. In the present study, by using a fluorescent technique that allowed us to evaluate pH with a greater time resolution and for longer times, we determined that the resting pH of either acutely or chronically deprived cells is lower than that of cells continuously exposed to 10% serum. However, we did not observe any significant difference between chronically and acutely deprived cells in either the resting pH or in the time response to the different serum concentrations tested in this study. Important differences between the previous study and the present study were the length of the acute deprivation period (20 min previously versus 1.5-2 hr presently) as well as the fact that the previous study used cells that were suspended and not anchored to any surface. The results of both studies indicate that maintenance of a higher pH requires the continuous presence of serum and that removal of serum for as little as 20 min (previous study) causes the cells to have a lower pH. According to the present study, this drop in pH is maximal by 1.5-2.0 hr, when it reaches a value similar to that observed in chronically deprived cells (Fig. 4). The drop in pH in response to removal of serum occurs with very slow kinetics, and the new resting pH is similar to that observed after chronic serum deprivation. If we assume that Na⁺/H⁺ exchange is activated by phosphorylation (for a review, see Moolenar, 1986), the time of 1.5-2.0 hr to reach basal pH may be indic-
This difference suggests that cells are sensitized to serum stimulation upon prolonged deprivation for serum levels stoichiometrically activate intermediate activation taking place in 3.7% and 10% serum. These results therefore suggest that the intermediate pH

**Table 1. Analysis of slope and correlation between intra- and extracellular pH**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Stimulated (10%)</th>
<th>Acutely deprived</th>
<th>Chronically deprived</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>pHm range</td>
<td>7.03-7.48</td>
<td>7.00-7.38</td>
<td>6.92-7.42</td>
</tr>
<tr>
<td>pHm mean ± S.D.</td>
<td>7.22 ± 0.11</td>
<td>7.21 ± 0.09</td>
<td>7.21 ± 0.10</td>
</tr>
<tr>
<td>pHm max ± S.D.</td>
<td>7.05 ± 0.13*</td>
<td>6.98 ± 0.11</td>
<td>6.97 ± 0.14</td>
</tr>
<tr>
<td>Slope (mean ± S.D.)</td>
<td>0.51 ± 0.16</td>
<td>0.64 ± 1.60</td>
<td>0.99 ± 1.32</td>
</tr>
<tr>
<td>Intercept (mean ± S.D.)</td>
<td>2.56 ± 0.25</td>
<td>2.56 ± 0.22</td>
<td>-0.23 ± 0.21</td>
</tr>
<tr>
<td>r</td>
<td>0.497</td>
<td>0.505</td>
<td>0.700**</td>
</tr>
</tbody>
</table>

*BALB/c-3T3 cells growing chronically in 10% Nu serum (stimulated) and either acutely or chronically deprived were used for this study. Intra- and extracellular pH was determined as described under "Materials and Methods." N represents the No. of experiments, whereas "r" represents the correlation coefficient of Pearson between intra- and extracellular pH. Statistical analysis was done by using the Student t-test for compared data. **P < .05 for chronically serum deprived cells.***

Fig. 5. Intra- versus extracellular pH of chronically serum-deprived (triangles) or serum-stimulated (circles) BALB/c-3T3 cells in the presence of 0.2% or 10% Nu serum, respectively. Each datum represents the mean of at least ten different time points taken in a period of 60. Few are available for both pHm and pH",. Plotting of the straight line was done by the method of least squares. Linear regression and correlation coefficient analysis showed that r = 0.497 for chronically deprived cells (triangles), and an r = 0.497 for stimulated cells (circles). Slope for chronically serum-deprived cells is a continuous line, whereas the discontinuous line represents the slope corresponding to stimulated cells. Differences between slopes are not statistically significant (see Table 1).

This is a graded phenomena (Fig. 3). We have previously confirmed using another biochemical approach since it may have significance to the "in vivo" responsiveness of cells to growth-promoting agents.

BALB/c-3T3 cells are able to regulate their pHm when they are chronically exposed to 10% serum or are only acutely deprived of serum. The steeper slope as well as the higher correlation coefficient found in chronically deprived cells, as opposed to cells in serum, support the hypothesis that cells deprived of serum for 48 hours lose their ability to regulate pHm in response to variations in pHm. The slopes of the chronically deprived cells and the stimulated cells are not significantly different. This is probably due to the fact that these data were collected over several months, with anticipated nonsystematic errors, as discussed below. However, for a given set of conditions (e.g., pHm 7.15), there are significant differences, which can be measured in a single preparation (Figs. 1, 3). As shown in Figure 5, the upward trend of data with a larger slope obtained from chronically serum-deprived cells, as compared to a steeper slope found in cells chronically exposed to 10% serum, suggest that at some pHm, such as 7.4, the slopes will coincide. We have consistently determined that there is no increase in pHm in response to serum addition in either acutely or chronically serum-deprived cells at a pHm of 7.4 data not shown.

A low correlation coefficient for the data in Figure 5 and Table 1 could come from perfect nonlinear data or imperfect linear data. Hence, we must consider the possibility that the low correlation coefficient found in

cells do not differ significantly in their response times to serum (Table 2). If we can assume that the kinetics of the pHm changes are dependent on the increased activity of Na"/H" exchangers, then the above data would suggest that acutely deprived cells have just as many activatable Na"/H" exchangers as do chronically deprived cells. This argument further implies that the loss of pHm regulatory capability by chronically deprived cells is not correlated to a loss in functional Na"/H" exchangers, and therefore must be coupled to loss of another pHm-regulatory pathway, possibly NaHC03/HCl exchange (L'Allemain et al., 1985; Challet et al., 1986; Boron, 1986). It is not known at this time whether this sensitization is paralleled by changes in the affinity of cell surface receptors for serum components or whether there are differences in the coupling between serum component receptors and the Na"/H" exchangers. This conclusion must be confirmed using another biochemical approach since it may have significance to the "in vivo" responsiveness of cells to growth-promoting agents.

addition of submaximal doses of serum to serum-stimulated cells. Differences between slopes are not statistically significant. Therefore, the above data would suggest that acutely deprived cells have just as many activatable Na"/H" exchangers as do chronically deprived cells. This argument further implies that the loss of pHm regulatory capability by chronically deprived cells is not correlated to a loss in functional Na"/H" exchangers, and therefore must be coupled to loss of another pHm-regulatory pathway, possibly NaHC03/HCl exchange (L'Allemain et al., 1985; Challet et al., 1986; Boron, 1986). It is not known at this time whether this sensitization is paralleled by changes in the affinity of cell surface receptors for serum components or whether there are differences in the coupling between serum component receptors and the Na"/H" exchangers. This conclusion must be confirmed using another biochemical approach since it may have significance to the "in vivo" responsiveness of cells to growth-promoting agents.
the stimulated and acutely deprived populations could be due to a greater variability in pH\textsuperscript{in} at a given pH\textsuperscript{ex} (imperfect data), as compared to the chronically deprived cells. We feel that this is not the case, since the pH\textsuperscript{in} of BALB/c-3T3 cells continuously exposed to 10% serum exhibits little population variability, whereas cells subjected to serum deprivation have a broader range of pH\textsuperscript{in} than do chronically stimulated cells (Gillies et al., 1987; Bright et al., 1987). This indicates that there is more heterogeneity of pH\textsuperscript{in} in serum-deprived cells, as compared to stimulated cells, and thus supports our contention that the low correlation coefficient observed in Table 1 for stimulated and acutely deprived cells arises from a true nonlinearity of the data and not from a greater heterogeneity in the pH\textsuperscript{in} of these populations.

There is, however, a large variability in the data for both deprived and stimulated cells (Fig. 6). We feel that this is caused by nonsystematic errors inherent in the system and does not reflect heterogeneity of pH\textsuperscript{in} values. The major parameters causing errors are temperature and calibration. As we discussed in greater detail in our previous report (Giuliano and Gillies, 1987), the pH\textsuperscript{in} of pyranine is temperature sensitive, increasing by 0.036 pH unit/°C. This indicates that the temperature must be maintained to ±1.5°C for pH\textsuperscript{in} measurements to be accurate to ±0.05 pH unit. Errors caused by calibrations are most problematic, since no real basis for comparison exist. We must assume that the nigericin/high K\textsuperscript{+} treatment completely breaks down the transmembrane pH gradient, that there is no significant Donnan potential in the presence of nigericin/high K\textsuperscript{+}, that the external pH is properly measured, and that the intracellular environment does not significantly affect the behavior of the dye. Nevertheless, these errors arising from inaccuracies in calibration and temperature are the same for all conditions and are not significantly different in magnitude from those of other methods (Roos and Boron, 1984; Nutcelli and Deaner, 1982; Bright et al., 1987; Gillies et al., 1987). These parameters can influence the observed magnitude and sensitivity of the pH\textsuperscript{in} change in response to variations in pH\textsuperscript{ex}. These errors are systematic within a single experimental preparation; therefore, the individual data points shown in Figure 5 will be affected by these errors, whereas the trends of the data, represented by the slope and correlation coefficient, will not.

A difference of 0.081 pH unit exists between cells in the presence or absence of serum at a pH\textsuperscript{ex} of 7.22. This difference is lower than those reported by other investigators, which range from 0.13 to 0.26 pH units (Schuldiner and Rozengurt, 1982; Cassel et al., 1983; L'Allemand et al., 1984; Moolenaar et al., 1984; Hesketh et al., 1985; Bright et al., 1987). A significant difference in these studies is that we used media containing 24 mM NaH\textsubscript{2}CO\textsubscript{3}. Another possible explanation for the discrepancy is that different pH\textsuperscript{in} values were used in these experiments. Since we demonstrate that pH\textsuperscript{in} is affected by pH\textsuperscript{ex}, reporting differences in pH\textsuperscript{in} are meaningless without knowing the various pH\textsuperscript{ex} values used in those studies. In the extreme case, our data predict, and we have observed, that the pH\textsuperscript{in} change will be virtually zero at pH\textsuperscript{ex} = 7.4. Since this pH\textsuperscript{ex} is close to that found in culture, it raises the possibility that the pH\textsuperscript{in} change caused by serum is physiologically insignificant.

These data also show that bicarbonate by itself does not necessarily prevent serum-induced acidification although it does seem to attenuate the response kinetics. Similar conclusions have been reached for other groups using 3T3 cells (Schuldiner and Rozengurt, 1982; Burns and Rozengurt, 1983; Lopez-Rivas et al., 1984; Hesketh et al., 1985). However, it should be reiterated that the pH\textsuperscript{in} change that occurs upon mitogenic activation is strongly dependent on pH\textsuperscript{ex}, and that bicarbonate can affect the degree of this sensitivity.

In summary, these observations indicate that prolonged serum deprivation of BALB/c-3T3 cells has an effect on the magnitude and sensitivity of the pH\textsuperscript{in} response to serum. We have also determined that serum-deprived cells have a consistently lower resting pH\textsuperscript{in} than cells in the presence of 10% serum, that this difference in pH\textsuperscript{in} is strongly dependent on pH\textsuperscript{ex}, and that there is no significant difference between the resting pH\textsuperscript{in} of acutely or chronically serum-deprived cells.

**ACKNOWLEDGMENTS**

The authors would like to thank Judith M. Sneider for her assistance in preparing cell cultures and Gloria M. Martinez for her technical assistance. This work was supported by NIH grant #GM-34656.

**LITERATURE CITED**


Cassel, D., Rothenberg, P., Zhugan, Y.X., Deusel, T.F., and Glaser, L. (1983) Platelet-derived growth factor stimulates Na\textsuperscript{+}/H\textsuperscript{+} exchange


SECTION 2.

Sphingosine inhibits phorbol 12-myristate 13-acetate-, but not serum-induced activation of Na⁺/H⁺ exchange in mammalian cells.

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Sphingosine Inhibits Phorbol 12-Myristate 13-Acetate-, but Not Serum-Induced, Activation of Na⁺/H⁺ Exchange in Mammalian Cells

ROBERT J. GILLIES,* RAUL MARTINEZ, JUDITH M. SNEIDER, AND PATRICIA B. HOYER

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Addition of serum to quiescent mammalian cells in culture initiates a series of events which culminates in DNA replication and cell division. One of the earliest events in this sequence of events is activation of Na⁺/H⁺ exchange, which can result in an increase in intracellular pH (pHᵣ). The regulation of this change in activity is not known. Since treatment of 3T3 cells with activators of protein kinase C (kinase C) can result in an increased pHᵣ, it has been hypothesized that serum stimulation of kinase C is responsible for activation of Na⁺/H⁺ exchange. Recently, sphingolipids have been discovered to inhibit kinase C both in vitro and in vivo. Therefore, we undertook the present study to ask whether or not inhibition of kinase C using sphingolipids prevents mitogen-induced alkalization in 3T3 cells. Our results indicate that activators of kinase C stimulate Na⁺/H⁺ exchange in normal human fibroblasts (BoGi), but not in mouse embryo (3T3) cells. Addition of serum to BoGi cells, on top of saturating doses of phorbol 12-myristate 13-acetate (PMA), results in a further cytoplasmic alkalization.

Furthermore, sphingosine prevents the PMA-induced increase in pHᵣ in BoGi cells, and phosphorylation of an 80 kDa protein in 3T3 cells, but not the serum-induced alkalization in either BoGi or 3T3 cells. These data indicate that activation of kinase C does not participate in the physiological activation of Na⁺/H⁺ exchange in human fibroblasts or mouse embryo cells by serum.

Most mammalian cells in culture require serum or associated components for sustained proliferation (Baserga, 1985). In the absence of serum, these cells will become quiescent. Re-addition of serum to these deprived cells initiates a sequence of events which culminates in DNA replication and cell division. One of the earliest events in this sequence is activation of Na⁺/H⁺ exchange, which can result in an increased intracellular pH (pHᵣ) (for review, see Moolenaar, 1986). An elevated pHᵣ is necessary for some of the later events of the activation sequence, such as increased protein synthesis (Pouyssegur et al., 1985) and initiation of DNA replication (Lucas et al., 1988). Activation of Na⁺/H⁺ exchange occurs in response to the addition of protein kinase C activators in a number of systems, but not in others (Burns and Rozengurt, 1983; Moolenaar et al., 1984; Varà et al., 1985; Grinstein et al., 1985; Paris and Pouyssegur, 1984; Hesketh et al., 1985). Many researchers have hypothesized that kinase C is a necessary and physiological transducer of the mitogen-induced activation of Na⁺/H⁺ exchanger in responsive cells (Burns and Rozengurt, 1983; Moolenaar et al., 1984; Paris and Pouyssegur, 1984; Pouyssegur et al., 1985; Varà et al., 1985; Grinstein et al., 1985; Hesketh et al., 1985). Recently, sphingolipids have been discovered to inhibit kinase C both in vitro (Hannun et al., 1986) and in vivo (Merrill et al., 1986; Wilson et al., 1986). This finding allows one the opportunity to inhibit kinase C during the mitogenic response.

We undertook the present study to ask whether or not inhibition of kinase C using sphingosine prevents the mitogen-induced alkalization. We have determined that activators of kinase C, such as phorbol esters, activate Na⁺/H⁺ exchange in normal human fibroblasts (BoGi) cells, but not in mouse embryo (3T3) cells. Furthermore, sphingosine prevents the phorbol ester induced increase in pHᵣ in BoGi cells, and endogenous phosphorylation of an 80 kDa protein in 3T3 cells, but not the serum-induced alkalization in either BoGi or 3T3 cells. These data indicate that protein kinase C is not involved in the serum-induced stimulation of Na⁺/H⁺ exchange in either cell type.

Received May 2, 1988; accepted November 15, 1988.

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MATERIAL AND METHODS

Cell culture

BALB/c-3T3 mouse embryo fibroblasts were obtained from American Type Culture Collection, Rockville, MD (ATCC CCL 163). They were cultured in Dulbecco's modified Eagle's medium (DMEM-Gibco, Grand Island, NY) supplemented with 10% Nu-serum; a synthetic mixture of growth factors supplemented with 25% characterized calf serum (Collaborative Research, Bedford, MA, and Hyclone, Logan, UT). The initial inoculum as received were frozen in DMEM containing 10% dimethylsulfoxide (DMSO), and 20% Nu-serum, at a density of 1 x 10^6 cells per freezing ampule. The cells were recovered in 75 cm^2 tissue culture flasks and passed twice at a density of 10^5 cells/dish in DMEM plus 10% Nu-serum. BoGi cells were grown from a subcutaneous pathological specimen obtained during minor surgery on a normal, healthy, good-looking, and intelligent adult male volunteer. Cells were grown out from the 1 g specimen in DMEM in 75 cm^2 and have a typical fibroblast morphology. After this initial inoculum, cultures were passaged 75 times before exhaustion. Cells used in this study were in passage 15-24. Subsequent culture of BoGi cells was as described for 3T3 cells. NIH-3T3 cells were a gift from Dr. G. Duester (Dept. Biochem, Col. St. Univ.), Swiss 3T3 cells were obtained from ATCC (ATCC CCL 92).

Intracellular pH determinations

Intracellular pH was determined from the fluorescence of pyranine as described in Giuliano and Gilles (1987). Briefly, cultures were washed with buffer containing 5 mM glucose, 2 mM glutamine, 25 mM HEPES in HBSS, pH 7.15 (CSB); scrape-loaded with 1 mM pyranine (McNeil et al., 1984); and washed and inoculated onto coverslips. After 1.5 hours at 37°C, coverslips were placed in cuvette and perfused with buffer or media at 37°C. Intracellular pH was determined by 465/405 fluorescence excitation ratio at an emission of 514 nm using a SLM 4800 fluorometer. In all experiments, the extracellular pH was continuously recorded with micro pH electrodes (Microelectrodes, Inc., Londonerry, NH) in the fluorometer cuvette.

Phosphorylation studies

As a measure of kinase C activity we examined the phosphorylation of p80, which is a well-characterized substrate of kinase C (Rozengurt et al., 1983; Albert et al., 1986; Blackshear et al., 1986; Smulke et al., 1986). Briefly, the cultures were washed twice with CSB and pre-incubated for 1 hour in the presence or absence of 10 mM phosphatidylserine. The cells were then incubated with media containing 200 mM orthophosphate (50 mM sodium orthophosphate) at 37°C for 1 hour to label the endogenous ATP pool. Sphingosine-treated cells were then incubated with 10 mM p80, 50 mM sodium orthophosphate, and 200 mM orthophosphate for 1 hour at 37°C. The reaction was stopped by placing the plates on ice, removing the medium, and immediately washing with 4 ml ice-cold CSB. The cells were then scraped off the plate with a rubber policeman and washed with an additional 4 ml cold CSB. The cell suspension was centrifuged (900 g, 4°C) and the pellet dissolved in 200 µl of 0.1 M phosphate buffered saline (PBS), 2% SDS, pH 7.0. Samples were solubilized by placing tubes in a boiling water bath for 2 minutes prior to resolution by gel electrophoresis.

SDS-polyacrylamide gel electrophoresis and autoradiography

Cellular proteins were separated by SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) as previously described (Hoyer and Niwander, 1986). The system employed a linear gradient (8-20%) of polyacrylamide with a 4% stacking gel. The gels were Coomassie stained, destained, fixed, and dried. Visualization of 32P incorporation was accomplished by autoradiography with Kodak XAR-5 film (Rochester, NY). Cellular protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Chemicals

Phorbol 12-myristate 13-acetate (PMA) was obtained from both Calbiochem (LaJolla, CA) and from Sigma (St. Louis, MO). Phorbol dibutyrate (PdBu) and dioctanoyl glycerol (DOG) were obtained from Sigma. All of these compounds were solubilized to concentrations of 1-10 mM in 95% ethanol. Solutions were stored at -20°C in the dark until used and were diluted directly in the test media. Sphingosine was obtained from both Calbiochem and Sigma and prepared by solubilization in 95% ethanol and mixing this in a 1:1 molar ratio with fatty acid free BSA (Sigma) at a concentration of 1 mM in CSB. This mixture was either stored at -20°C or directly diluted into the test media. Chemicals used for electrophoresis were purchased from Sigma. 32P orthophosphate was obtained from New England Nuclear (Boston, MA).

RESULTS

Addition of serum to quiescent BALB/c-3T3 cells causes an increase in pHm from 6.9 to 7.15, as illustrated in Figure 1A. The magnitude of this alkalization is dependent on a number of factors, including the extracellular pH, and the concentrations of both serum and bicarbonate (Martinez et al., 1988). It is generally accepted that the increase in pHm is caused by activation of a Na^+/H^+ exchange mechanism (Moolenaar, 1986). This activation is illustrated in Figure 1B-D, where the pHm of BALB/c-3T3 cells is shown in response to intracellular acid loads (50 mM sodium acetate). Note that the kinetics of pHm recovery are much slower in the presence of serum at submitogenic doses (Fig. 1B), than at mitogenic doses (Fig. 1C). The kinetics of re-alkalinization is illustrated in Figure 1D, where the rate of pHm recovery is expressed as a function of the pHm for cells in the presence of low and high concentrations of serum. These data indicate that serum induces a change in the Na^+/H^+ exchange mechanism which makes it active at higher pHm values (for a review, see Moolenaar, 1986). In addition, this serum-induced activation is inhabitable by amiloride and is dependent on external Na^+ (unpublished observations).

Alkalization of pHm and/or activation of Na^+/H^+ exchange mechanisms is dependent on the extracellular concentrations of sodium and bicarbonate. The effect of serum on the Na^+/H^+ exchange mechanism is illustrated in Figure 1E, where the rate of pHm recovery is expressed as a function of the rate of pHm recovery for cells in the absence of serum. The results indicate that serum enhances the rate of pHm recovery, but does not affect the rate of pHm recovery at the lower concentrations of serum. The results also indicate that the effects of serum on the Na^+/H^+ exchange mechanism are dependent on the extracellular concentrations of sodium and bicarbonate. The effect of serum on the Na^+/H^+ exchange mechanism is illustrated in Figure 1F, where the rate of pHm recovery is expressed as a function of the rate of pHm recovery for cells in the absence of serum. The results indicate that serum enhances the rate of pHm recovery, but does not affect the rate of pHm recovery at the lower concentrations of serum. The results also indicate that the effects of serum on the Na^+/H^+ exchange mechanism are dependent on the extracellular concentrations of sodium and bicarbonate.
Sphingosine inhibition of Na⁺/H⁺ exchange

**Figure 1.** The effect of serum on pHᵢ and the activity of Na⁺/H⁺ exchanger in BALB/c3T3 cells. A: Response of pHᵢ to serum addition in cells deprived of serum for 2 hours (acutely deprived). Perfusion was begun with CBSt containing Na-serum at a concentration of 2% acetyl and was exchanged for CBSt containing Na-serum at 10% acetyl (1% CSB) at the time indicated. B: pHᵢ recovery from serum stress induced acidification in acutely serum-deprived cells. Perfusion was begun in 0.2% CSB and, at the time indicated, the buffer was exchanged to 0.2% CSB containing 50 mM sodium acetate (306 mOsM/kg). C: pHᵢ recovery in the presence of 10% Na-serum. All other conditions were identical to those described (B). D: Relationship between rate of pHᵢ recovery and pHᵢ in acutely serum-deprived cells (circles) and cells in 10% Na-serum (squares). These data were obtained by plotting the derivatives of data similar to those shown in Figure 1B as a function of the pHᵢ (see Moolenaar, 1986). Lines represent linear least squares regression of the data.

Exchange has been reported to occur in response to kinase C activators. As illustrated in Figure 2C, the pHᵢ of BoGi cells increases in response to the addition of PMA. Similar results have been obtained a number of times with concentrations from 1 to 500 nM PMA, with the maximum effect occurring at concentrations above 20 nM. In contrast, the data illustrated in Figure 2B demonstrate that PMA has no effect on the pHᵢ of BALB/c-3T3 cells. This experiment has been repeated, with identical results, using a number of experimental variables. We have repeated these experiments using three types of 3T3 cells: BALB/c, Swiss, and NIH. We have repeated these experiments using PMA, PdBu, and DOG at concentrations ranging from 2 nM to 5 μM. We have added PMA and PdBu in the presence of agents which will raise intracellular Ca²⁺ levels, such as A23187 (Moolenaar et al., 1986) and DEAE dextran (Caldwell et al., 1986) in the expectation that the cellular Ca²⁺ levels might have been so low as to preclude activation of kinase C with any level of phorbol. We have also determined that kinase C activators have no effect on the recovery of pHᵢ to an acidic load. The results of these experiments consistently indicate that activators of kinase C are unable to stimulate Na⁺/H⁺ exchange in BALB/c-3T3 cells under a wide variety of conditions.

Before adding sphingosine to our cells in the fluorescence cuvette, we first determined the effect of this lipid on the viability of 3T3 and BoGi cells, because it has been reported to be cytotoxic to Chinese hamster ovary (CHO) cells. In our hands, sphingosine at concentrations from 1 to 100 μM did not affect cell viability during the time required for pHᵢ studies (up to 5 hours), as evaluated by the exclusion of trypan blue. However, after 48 hours under culture conditions, sphingosine is cytotoxic to both 3T3 and BoGi cells at concentrations above 2 μM (Fig. 3). The Km of kinase C for sphingosine is 2–4 μM (Hannun et al., 1986; Merrill et al., 1986), suggesting that the effect that we are observing is mediated through inhibition of kinase C. We have obtained virtually identical data from a number of other cell types, such as CHO, Ehrlich ascites tumor, Swiss- and NIH-3T3 cells (data not shown).

Since sphingosine is not cytotoxic within our experimental time frame, we next tested whether sphingosine inhibits activation of kinase C phosphotransferase activity by PMA in 3T3 cells. This was determined by monitoring the kinase C-induced phosphorylation of a polypeptide with an apparent molecular weight of 60 kDa (Ronengart et al., 1985; Albert et al., 1986; Blackshear et al., 1986; Isaac et al., 1986). Our data indicate that 10 μM sphingosine causes nearly complete inhibition of PMA-induced 80 kDa phosphorylation. Other proteins demonstrating inhibition by sphingosine of PMA-stimulated phosphorylation were identified with apparent molecular weights of 42 kDa and 23 kDa (Fig. 4).

The PMA-induced alkalization of BoGi cells is inhibited by sphingosine, as illustrated in Figure 2D. However, inhibition of this phorbol-sensitive alkalization is not sufficient to inhibit the pHᵢ change which occurs upon subsequent addition of serum. Similarly, sphingosine has no effect on the serum-induced pHᵢ response in BALB/c-3T3 cells, as illustrated in Figure 2A. These experiments have been repeated with
Fig. 2. The effect of sphingosine and PMA on the pH of BoGi and BALB/c-3T3 cells. A: BALB/c-3T3 cells. B: BoGi cells. At sphingosine does not inhibit the serum-induced alkalinization in BALB/c-3T3 cells. In this experiment, perfusion was begun in 0.2% CSB. At 12 minutes, perfusate was changed for one containing 0.2% CSB plus 10 nM sphingosine. After 40 minutes, perfusate was changed to 0.2% CSB containing PMA at 500 nM. B: PMA does not increase pH in BALB/c-3T3 cells. Perfusion was begun in 0.2% CSB. At 15 minutes, perfusate was changed for 0.2% CSB containing PMA at a concentration of 100 nM. After 30 minutes in this buffer, perfusate was changed to 0.2% CSB containing PMA at 500 nM. After 30 minutes, perfusate was changed for one containing 500 nM PMA and 10% CSB. C: PMA and serum both stimulate an increase in pH in BoGi cells. Conditions in this experiment were identical with those described in B, except that BoGi cells were used and the 100 nM PMA increased was eliminated. D: Sphingosine inhibited alkalinization in BoGi cells. Conditions in this experiment were identical with those described in B, except that sphingosine was added to the perfusate. 

Fig. 3. Effect of sphingosine on cell number. BALB/c-3T3 and BoGi cells were plated in 24-well culture plates (Flow Labs) in DMEM with 10% FCS. After 12 hours of culture, sphingosine was added to the wells at the concentrations indicated. After 48 hours in the presence of sphingosine, the cultures were fixed, stained, and absorbance was read, as described in Gillies et al. (1986). O.D. is directly proportional to cell number. Data represent mean and standard deviation. Lines drawn represent third-order polynomial fit to data (Sigma Plot-Jandel, Inc.).

Fig. 4. Sphingosine inhibition of 80 KDa phosphorylation following PMA treatment of BALB/c-3T3 cells in monolayer growth. BALB/c-3T3 cells were plated at 1 x 10^6 cells/100 mm petri dishes for 48 hours. Prior to the experiment, cells were washed and sphingosine 100 nM was added to half the cultures. Following 1 hour of incubation, [32P]PO_4 was added to each plate for an additional 1 hour. At that time, 20 nM PMA was added for 5 minutes, as indicated. Lane 1: Control. Lane 2: PMA. Lane 3: Sphingosine. Lane 4: PMA plus sphingosine. Samples were processed for protein separation and visualization of [32P]incorporation as described in Materials and Methods. Note: The amount of protein recovered in the sample applied to lane 3 was not as great as that recovered in the other samples determined by visual inspection of Coomassie blue staining; data not shown.

Three batches of sphingosine from two different sources, with concentrations as high as 100 nM, and with pre-incubation times of up to 5 hours. None of these treatments show any inhibition of either the serum-induced pH change, or the kinetics of recovery to an acid load. These data indicate that inhibition of...
kinase C does not affect the ability of serum to activate Na"+ /H+ exchange by at least one and his experiment, we expect the kinase serum, and that inhibition of kinase experiments. Suspension and reattachment of 3T3 cells to cytoplasmic cells to can induce an increase in pathway that is distinct from that induced by factor (1985) using 3T3 cells, although, in that case, it was derived growth factor able to activate Na +/H + exchange, such as such as phorbol esters prior to activation of kinase C in both systems, yet it was not able to inhibit the serum-induced activation of Na"+ /H+ exchange.

DISCUSSION

Activators of kinase C do not stimulate Na"+ /H+ exchange in BALB/c-3T3 cells. Similar results have been reported in other cell lines (Magnaldo et al., 1986; Macara, 1986; Bestrepe et al., 1987). However, the present results are discrepant with other literature reports that have found an effect of PMA on the pHm of 3T3 cells (Paris and Pouyssegur, 1984; Grinstein et al., 1985). There are a number of possible reasons for this difference. One possible reason is that our cells are cultured using Nu-serum, while other researchers have supplemented their culture media with fetal bovine serum. Another major difference between our cultures and those from others is that our cells are scrape-loaded 1.5-2.0 hours prior to experiments. Suspension and reattachment of 3T3 cells has recently been shown to induce c-my and c-fos (Dike and Farmer, 1987), implying that oncogene induction might inhibit responsiveness to kinase C. However, this would not be a general phenomenon, since Na"+ /H+ exchange of similarly loaded cells (e.g., BoGi in this paper, DMSO-treated 3T3 in Martinez and Gilles, 1985) is able to respond to PMA.

It is interesting to note that the addition of serum to BoGi cells, on top of saturating doses of PMA, results in a further cytoplasmic alkalization. This phenomenon is similar to an observation made by Hesketh et al. (1985) using 3T3 cells, although, in that case, it was unclear whether the level of PMA used gave a maximal pH response. At the levels of PMA that we have used in this experiment, we expect the kinase C in the cell to be fully activated, since the Km of kinase C for PMA is about 7 nM (Nishizuka, 1984), and control experiments in our lab have indicated that the pH response of these cells to PMA is maximal at 20 nM. Therefore, serum can induce an increase in pHm above and beyond that which is inducible by PMA, suggesting that serum is able to activate Na"+ /H+ exchange by at least one pathway that is distinct from that induced by PMA.

The above data suggest that kinase C does not participate in the physiological activation of Na"+ /H+ exchange by serum. This hypothesis is supported by the observation that kinase C activators stimulate cytoplasmic alkalization in BoGi cells, that this stimulation is not as large as that observed with serum, and that inhibition of kinase C with sphingosine does not inhibit the serum-induced alkalization in either 3T3 or BoGi cells.

Serum contains a number of compounds which are able to activate Na"+ /H+ exchange, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). PDGF activation involves the release of diacylglycerol and leads to the activation of kinase C, whereas EGF activation does not (Vara et al., 1985; Hesketh et al., 1985; Moolenaar, 1986). Hence, our data suggest that the serum component which activates Na"+ /H+ exchange in these cells is EGF-like and not PDGF-like. Nevertheless, BoGi cells do alkalize in response to PMA. We hypothesize that this may be the result of a promiscuous phosphorylation of Na"+ /H+ exchange by this phorbol-activatable kinase C in this system. Since, it appears from these results and those from others (Burns and Rozengurt, 1985; Paris and Pouyssegur, 1984; Moolenaar et al., 1984; Vara et al., 1985; Grinstein et al., 1985; Hesketh et al., 1985), that activation of kinase C, and hence phosphorylation, has the potential to activate Na"+ /H+ exchange, it is likely that some kinase or kinases besides kinase C are physiologically active in the induction of cytoplasmic alkalization by serum. Since 'kinase C' is actually a family of enzymes, it is also possible that the kinase activated by serum is a PMA- and sphingosine-insensitive isoform of 'kinase C'.

ACKNOWLEDGMENTS

The authors would like to thank Mark Stewart, Kenneth A. Giuliano and Gloria M. Martinez for their technical assistance in the early stages of this work, and especially Steve Yemm for providing the human fibroblast-containing specimen. This work was supported by NIH grant ROI GM40337 to R.J.G., BRSG #2507 RR05675 to P.B.H., and NIGMS Shared Instrumentation grant RR04183 for purchase of SLM 4800 fluorometer.

LITERATURE CITED


SECTION 3.

Dimethylsulfoxide modifies the sensitivity of BALB/c-3T3 cells to the activation of Na⁺/H⁺ exchange by phorbol esters.

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Dimethylsulfoxide Modifies the Sensitivity of BALB/C-3T3 Cells to the Activation of Na⁺/H⁺ Exchange by Phorbol Esters

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In a companion paper (Gillies et al.; J. Cell. Physiol. 139:124–129, 1989) we show that phorbol esters (PEs) are unable to stimulate Na⁺/H⁺ exchange in BALB/c-3T3 cells under a wide variety of conditions. The Na⁺/H⁺ exchangers of a number of other cell types are also not responsive to PEs yet have been rendered responsive by treatment with agents such as dimethylsulfoxide (DMSO). We undertook the present study to evaluate whether the treatment of BALB/c-3T3 cells with DMSO will induce modifications in the sensitivity of these cells to activation by PEs. The present study indicates that a 3–5 day exposure of BALB/c-3T3 cells to 1.25% DMSO leads to changes in the sensitivity of these cells to the activation of Na⁺/H⁺ exchanger by PEs. These changes in sensitivity were apparent at day 3 and maximal at day 5. Non-tumor-promoting analogues of PEs do not activate Na⁺/H⁺ exchange, suggesting that the effect is mediated through kinase C. Sphingosine prevents PEs, but not serum-induced alkalinization. However, the half-time of the intracellular pH (pHᵢ) response to serum was increased by sphingosine, suggesting that kinase C participates in, but is not required for, the serum induced activation. Since DMSO does not induce any apparent morphological change, the change in sensitivity of Na⁺/H⁺ exchange to PEs is not likely to be related to differentiation, but may be associated with structural changes in the Na⁺/H⁺ exchanger and/or changes in isoforms of kinase C which recognize the exchanger as a substrate.

Recent data from our lab have shown that activators of kinase C are unable to stimulate Na⁺/H⁺ exchange in BALB/c-3T3 cells under a wide variety of conditions (Martinez and Gillies, 1987; Gillies et al., 1989a). Similar results have been reported in other cell lines (Magnaldo et al., 1986; Macara, 1986; Restrepo et al., 1987). However, our results are discrepant with other literature reports that have shown an effect of phorbol, 12-myristate 13-acetate (PMA), on the activity of Na⁺/H⁺ exchange of 3T3 cells (Burns and Rozengurt, 1983; Paris and Pouysegu, 1984; Hesketh et al., 1985; Grassstein et al., 1985; Vera et al., 1985; Moelenaar, 1986). Therefore, we examined conditions which might sensitize 3T3 cell Na⁺/H⁺ exchange to activation by serum.

Na⁺/H⁺ exchange in undifferentiated HL-60 promyelocytic cells is not affected by PE treatment. However, DMSO-induced granulocytic differentiation of HL-60 cells causes their Na⁺/H⁺ exchange to become responsive to kinase C activators (Leftwich et al., 1987; Restrepo et al., 1987). We undertook the present study to evaluate whether the treatment of BALB/c-3T3 cells with DMSO induces a similar modification in the PE-dependent activation of Na⁺/H⁺ exchange. Our present results indicate that treatment of BALB/c-3T3 cells with DMSO for 3–5 days induces changes in the sensitivity of cells to PMA stimulation. The resting pHᵢ was not affected by DMSO-treatment. Inactive analogues of phorbol do not induce cytoplasmic alkalinization, suggesting that the effect is mediated through kinase C. Furthermore, as we found before with human fibroblasts (Gillies et al., 1989a), sphingosine effectively prevented the pHᵢ increase induced by PMA, but not by serum.

MATERIALS AND METHODS

Cell culture

BALB/c-3T3 mouse embryo fibroblasts were cultured and prepared for fluorescence as described in Gillies et al. (1989a). DMSO-treated cells were cultured in media containing 1.25% dimethylsulfoxide (DMSO) (Sigma Chemical Co.; St. Louis, MO) for up to 7 days. At the times indicated, these cells were scrape-loaded with pyranine and prepared for fluorometry in the presence of 0.2% Nu-serum in cell suspension buffer (CSB) (Gillies et al., 1989a). DMSO-treatment of BALB/c-3T3 cells does not induce any apparent morphological changes. On the other hand, DMSO does induce an arrest of cell proliferation after 48 hours and a decrease in cell volume (data not shown), consistent with other

Received May 2, 1988; accepted November 15, 1988.

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literature reports (Zucker et al., 1983; Costa-Cancille et al., 1987). Viability was assessed by trypan blue exclusion and remained greater than 90%.

**pH determinations**

Intracellular pH was determined by fluorescence of intracellular pyranine (Giuliano and Gillies, 1987; Gillies et al., 1989a). For all experiments, an external pH (pHout) of 7.1 was used, and was continuously recorded with mini-pH electrodes (Microelectrodes, Inc., Londonederry, NH) in the fluorometer cuvette.

**RESULTS**

Figure 1A shows that untreated, acutely serum-deprived BALB/c-3T3 cells, continuously perfused with 0.2% Nu-serum in bicarbonate-free CSB (pH 7.1), have a resting pHin of 6.65. Addition of 10% Nu-serum to these cells results in an increase in pHin which is due to the activation of Na+/H+ exchange (for a review, see Moolenaar, 1986). As shown in Figure 1B, these untreated cells do not respond with an increase in pHin by adding PMA at either 10 nM or 500 nM, but they do respond to the addition of 10% Nu-serum within the same experiment. These experiments have been done a number of times (Martinez and Gillies, 1987; Gillies et al., 1989a). In contrast, after 3 days of DMSO-treatment, BALB/c-3T3 cells respond to the addition of PMA with a slight, but significant increase in pHin (Fig. 2A; Table 1). Figure 2A also shows that addition of the non-tumor-promoting 4-a phorbol does not result in a cytoplasmic alkalinization, suggesting that the effect is mediated by kinase C (Grinstein and Furuya, 1986). Furthermore, addition of Nu-serum to these cells results in a further increase in pHin. Continued incubation of the cells with DMSO for 5 days leads to a maximum response in cytoplasmic alkalinization induced by PMA. The increase in pHin induced by 500 nM PMA after 5 days of treatment was similar to that caused by the addition of 10% Nu-serum (Fig. 2B; Table 1).

Cells exposed to DMSO for 1 or 2 days did not increase pHin in response to the addition of PMA. Similar results were obtained by using phorbol dibutyrate or dioctanoylglycerol at concentrations ranging from 5 nM to 1 μM (data not shown). In control studies, we observed that perfusion of either untreated or DMSO-treated cells with 1.25% DMSO in CSB plus 0.2% Nu-serum do not result in changes in pHin, at any of the times tested. Comparison of the resting pHin in both untreated and treated cells shows no significant differences between these cell populations (Table 1).

An alternative way to test for the specificity of this activation is shown in Figure 2C, where sphingosine, an inhibitor of kinase C (Hannun et al., 1986; Merrill et al., 1986; Wilson et al., 1986), prevents the increase in pHin induced by PMA, but not by Nu-serum in 5 day DMSO-treated cells.

The kinetics of response to serum-induced alkalinization was about twice as slow in these sphingosine- and DMSO-treated cells, as compared to untreated cells (Figs. 1A, 2C; Table 1). The half-time of response is measured as the time required for the cells to reach half of the new steady-state pHin in response to the stimulation by either serum or PMA. As shown in Table 1, there are no significant differences in the half-time of response to serum stimulation between these cell populations at any of the times tested. Therefore, DMSO treatment does not seem to modify the sensitivity of these cells to serum stimulation in terms of their response kinetics. On the other hand, the response time to PMA stimulation in cells treated with DMSO for 5 days indicates that the increase in pHin by PMA occurs with slower kinetics as compared with the fast increase in pHin induced by 10% Nu-serum (Table 1).

![Figure 1](image-url)
DMSO AND Na⁺/H⁺ EXCHANGE

DISCUSSION

The activation of Na⁺/H⁺ exchanger is illustrated in Figure 1A, where addition of 10% Nu-serum to acutely serum-deprived cells in the absence of HCO₃− causes an increase in pHᵢ from 6.65 to 6.68. The magnitude of this alkalization is dependent on a number of factors, including the pHᵢ, presence of bicarbonate, and the concentration of serum (Martinez et al., 1982; Gilles et al., 1986b). The activation of Na⁺/H⁺ exchanger has also been observed in a number of cell types after activation of kinase C with phorbol ester derivatives (Burns and Rozengurt, 1983; Paris and Pouyssegur, 1984; Moolenaar et al., 1984; Grinstein et al., 1985; Yaru et al., 1985; Hesketh et al., 1985). In contrast, our data show that PMA has no effect on the pHᵢ of BALB/c-3T3 cells (Fig. 1B) (cf. Gilles et al., 1989a).

However, treatment of BALB/c-3T3 cells with DMSO for 3 days "sensitizes" these cells to PMA stimulation. The increase in pHᵢ induced by PMA is probably due to an activation of kinase C, since 4-alpha phorbol (Grinstein and Furuya, 1986) does not affect pHᵢ (Fig. 2A), and sphingosine inhibits PMA induced alkalization (Fig. 2C). Although sphingosine does not inhibit the serum-induced pHᵢ change, it does increase the half-time of the response in DMSO-treated cells. Table 1 shows that the half-time of response to serum in DMSO-treated cells occurs with faster kinetics than in cells treated with sphingosine (Fig. 1A, 2C). Sphingosine does not modify the time-response to serum in the absence of DMSO. Therefore, it appears that treatment of BALB/c-3T3 cells with DMSO not only modifies the sensitivity of the Na⁺/H⁺ exchanger to PMA stimulation, but also to sphingosine inhibition, and further supports the hypothesis that kinase C is involved in the regulation of Na⁺/H⁺ exchanger in the DMSO-treated cells. If we assume that the Na⁺/H⁺ exchanger can be activated through phosphorylation by kinase C (Moolenaar et al., 1983; Grinstein et al., 1985) this relative decrease in the velocity of alkalization caused by sphingosine may represent the proportion of the serum-activated pathway in DMSO-treated cells which utilizes kinase C.

The observed changes in sensitivity of BALB/c-3T3 cells induced by DMSO do not affect the exchanger...
under resting physiological conditions. Resting pHm was similar in both treated and untreated cells (Figs. 1A, 2A; Table 1). Moreover, measurement of pHm during the period of 1 to 7 days of DMSO treatment did not reveal any changes in either resting pHm or in the sensitivity of these cells to serum stimulation (Table 1 and data not shown). Assuming that the kinetics of the pHm changes are dependent on the summed activity of Na+/H+ exchangers, then the above data would suggest that untreated cells have just as many activatable Na+/H+ exchangers as do treated cells.

The sensitivity of BALB/c-3T3 cells to PMA stimulation is not affected during the first 7 days of DMSO treatment. Similar observations have been made in HL-60 cells (Leftwich et al., 1987; Restrepo et al., 1987). The mechanism(s) that lead to the functional and/or structural change(s) induced by DMSO are unknown. If it is caused by some type of covalent modification, its delayed appearance would suggest that the activation of the catalyzing enzyme occurs after a long sequence of events. In HL-60 cells it has been observed that DMSO induces a threefold increase in kinase C activity (Zylber-Katz and Glazer, 1985; Lane et al., 1986). Furthermore, Coussens et al. (1986) have shown that there is a family of kinase C-related proteins. It is possible that DMSO treatment induces a shift from an isofrom of kinase C which does not recognize the Na+/H+ exchanger as a substrate to one which does. It is also possible that DMSO induces structural changes in the Na+/H+ exchanger (Costa-Canellie et al., 1987). Another interesting possibility is that DMSO induces a link needed to transduce kinase C stimulation into Na+/H+ exchange activation. We are currently exploring whether or not this link involves the phosphorylation of kinase C or if there is a GTP-dependent phosphorylation of the exchanger by other kinase(s).

**ACKNOWLEDGMENTS**

The authors would like to thank Dr. A. Young Woody for the use of the fluorometer, to James E. Olson for his helpful discussions, to Judith M. Sneider for her assistance in preparing cell cultures and to Gloria M. Martinez for her technical assistance. This work was supported by NHL grant RO1GM40337 to R.J.G. The SLM 4800 fluorometer used in this study was purchased through grant RO14151 from the NIGMS Shared Instrumentation Program.

**LITERATURE CITED**


Moleman, W.H., Tien, R.Y., van der Saag, P.T., and de Laat, S.W.


SECTION 4.

Regulation of intracellular pH in BALB/c-3T3 cells: Bicarbonate raises pH via NaHCO$_3$/HCl exchange and attenuates the activation of Na$^+$/H$^+$ exchange by serum.

March 25, 1991

Editorial Office
American Society for Biochemistry and Molecular Biology, Inc.
Attn.: Editorial Office
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Dear Sirs:

As a part of my Ph.D. dissertation entitled "The Role of Intracellular pH and Calcium in the Regulation of Cellular Functions", I would like to append an article that I have already published in J. Biol. Chem. 266:1551-1556, 1991. Therefore, I am requesting your permission to reproduce it.

Thank you for your assistance in this matter.

Sincerely,

Raúl Martínez-Zaguiláu

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The University of Arizona Colleges of Medicine, Nursing, Pharmacy, and School of Related Professions
University Medical Center and The University Physicians
Regulation of Intracellular pH in BALB/c 3T3 Cells

Robert J. Gilless and Raul Martinez-Zaguilan
From the Department of Biochemistry, University of Arizona Health Sciences Center, Tucson, Arizona 85724

There is abundant evidence implicating a role for intracellular pH (pH\textsubscript{i}) in the proliferative response of many cells to mitogenic agents. In mammalian cells, pH\textsubscript{i} is generally regulated by two systems: Na\textsuperscript{+}/H\textsuperscript{+} exchange and HCO\textsubscript{3}{-} transport. Activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange is one of the earliest responses of mammalian cells to mitogens. In the absence of HCO\textsubscript{3}{-}, this activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in the presence of HCO\textsubscript{3}{-} attenuates the mitogen-induced Na\textsuperscript{+}/H\textsuperscript{+} efflux of the cell. This factor stimulates the activity on the HCO\textsubscript{3}{-} transport, which raises its affinity for Na\textsuperscript{+} (4, 5).

...Generally, mammalian cells regulate their pH\textsubscript{i} values with two different transport activities: amiloride-inhibitable Na\textsuperscript{+}/H\textsuperscript{+} exchange and stibine-inhibitable HCO\textsubscript{3}{-} transport (4, 5). Na\textsuperscript{+}/H\textsuperscript{+} exchange uses the Na\textsuperscript{+} gradient to drive the extrusion of H\textsuperscript{+}(6). Under basal conditions, this antiporter acts to maintain the pH\textsubscript{i} in a "set point" (7). Activation by mitogens results in an unknown modification of the transporters, as a function of its affinity, leading to an increased pH\textsubscript{i} (4). In the absence of HCO3\textsuperscript{-}, its activity has been reported to raise the pH\textsubscript{i}, prompting many researchers to postulate that

\[ \text{HCO}_3^- \text{ transport} \rightarrow \text{pH}_i \text{ increases} \]

Intracellular pH (pH\textsubscript{i}) is a highly regulated and variable parameter in the physiology of mammalian cells. Changes in pH\textsubscript{i} have been reported to occur rapidly in response to a number of mitogens, such as serum or purified growth factors (1-3). These changes have been observed in a wide variety of cell types, including fibroblasts, epithelial, neuronal cells, and lymphocytes. Generally, mammalian cells regulate their pH\textsubscript{i} with two different transport activities: amiloride-inhibitable Na\textsuperscript{+}/H\textsuperscript{+} exchange and stibine-inhibitable HCO\textsubscript{3}{-} transport (4, 5). Na\textsuperscript{+}/H\textsuperscript{+} exchange uses the Na\textsuperscript{+} gradient to drive the extrusion of H\textsuperscript{+}(6). Under basal conditions, this antiporter acts to maintain the pH\textsubscript{i} in a "set point" (7). Activation by mitogens results in an unknown modification of the transporters, as a function of its affinity, leading to an increased pH\textsubscript{i} (4). In the absence of HCO3\textsuperscript{-}, its activity has been reported to raise the pH\textsubscript{i}, prompting many researchers to postulate that

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† To whom correspondence should be addressed.
Intracellular pH of 3T3 Cells

Materials and Methods

Cell Culture—BALB/c 3T3 mouse embryo fibroblasts were obtained from the American Type Culture Collection (ATCC CCL 163). They were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% Nu-serum (Collaborative Research Inc.). Passage 50, ML0, the initial inoculum as received was grown to 70% confluence in 300 cm² at which time the cells were frozen in Dulbecco's modified Eagle's medium supplemented with 10% DMSO and 20% Nu-serum, at a density of 1 x 10⁶ cells/freezing ampule. These cells were recovered every 6 weeks in 75-cm² tissue culture flasks and passed weekly at an inoculation density of 2 x 10⁶ cells/cm². Cells were subcultured for 2 weeks prior to being used for experiments. For experiments, cells were inoculated into 90-mm Petri dishes for pyrene experiments or Petri dishes containing 9 x 22-mm coverslips for experiments using SNARF-1 as a density of 10⁵ cells/dish in Dulbecco's modified Eagle's medium plus 10% Nu-serum. On the following day, these cultures were prepared for fluorescence determination of pH, as described below:

Fluorescence Measurements—Intracellular pH was determined by fluorescence of either intracellular pyranine (4-hydroxy-7-pyrone-1,3,6-trisulfonic acid) or SNARF-1 (N,N-dimethyl-1,5-di-(6-hexacobenzopyranosylsemispheroidofluorosulphonic acid) as indicated in the legend. In pyranine experiments, the dye was snap-loaded into cells as described by McNeill et al. (11), and subsequently treated as described fully by Giannoni and Gillies (14). Briefly, snap-loaded cells were allowed to recover at 37°C for 1 h, after which they were washed and allowed to settle for about 2 h prior to analysis. In HCO₃⁻ experiments, attachment took place in a 5% CO₂ atmosphere, whereas in HCO₃⁻ free experiments, attachment took place in a modulator buffer (Bel-Art plastic), equilibrated with air. In experiments using SNARF-1, the dye was loaded into cells grown on coverslips in the American Type Culture collection as described previously (15). Briefly, cells on coverslips were incubated for 2 h in CSB, followed by 30 min at 37°C in CSB (see below) containing 20 μM SNARF-1 (AM). After 30 min, the cells were rinsed three times with CSB and incubated a further 30 min to ensure complete ester hydrolysis. Fluorescence was determined using either an SLM-4600 or SLM-46000 spectrofluorometer outfitted with a flow-through device for sample perfusion (16). Two coverslips containing cells were mounted in the holder back to back and were continuously perfused at 0.2 ml/min. Sample temperature was maintained at 37°C by keeping both the water jacket and the perfusion medium at 37°C. The extracellular pH (pHₑₓ) was continuously monitored with a small pH electrode in the cuvette (Microelectrodes Inc., Londonderry, NH). All cells were not out of serum-containing media for a minimum of 3 h prior to analysis. Parallel studies have shown that 3 h is sufficient to develop complete pH-response to mitogens (data not shown).

Data Analysis—Half-maximal response time was determined as the time it takes for the pH to reach half of its final value. Beginning pH, and time were taken at the nadir, for realkalinization, or at the zenith, for reacidification experiments. Velocities were determined by least-squares fitting the recovery curve using a simple least squares fitting routine (MINITAB, MicroMath Software, Salt Lake City, UT) and calculating the dpH/dt at a pHₑₓ of 6.80.

Results

Effect of HCO₃⁻ or Nu-serum on Recovery of Cells to Cytosolic Acid Loads—The protonated form of acetate is much more membrane permeable than is the deprotonated form. Thus, addition of acetate to cells induces an acute acid load to the cytoplasm. This load is rapidly removed from the cytoplasm by either serum-stimulated Na⁺/H⁺ exchange or by anion (HCO₃⁻) exchange. Therefore, in the absence of Nu-serum and HCO₃⁻, both of these systems should be relatively inactive and recovery to acetate-induced acid load should be slow. This is illustrated in Fig. 1A. Addition of 10 mM acetate induces a decrease of pHₑₓ of 0.29 pH unit: from 6.92 to 6.63. The intracellular acid load ([H₄CO₃]₀), can be approximated by [50 mM/10 ΔHₑₓ - μt]³ in this case [H₄CO₃]₀ is about 15 mM. The intracellular buffering capacity can be estimated to be 15/0.29 = 52 mM H⁺/pH unit. Results from a number of similar experiments are presented in Table 1a. Under these conditions, the pHₑₓ recovery occurs with an initial velocity (V₀) of 7.3 x 10⁻⁸ pH unit/min. The H⁺ extrusion rate is therefore approximately 0.37 mM H⁺/min. Since the activity of H⁺ transport systems are dependent on pHₑₓ, H⁺ extrusion rates were calculated at a single pHₑₓ value (e.g. 6.80). In the presence of 24 mM HCÔ₃⁻, however, the pHₑₓ recovers much faster, as shown in Fig. 1B. In this case, 50 mM acetate also decreases pHₑₓ by 0.28 pH unit. However, since the starting (and final) pHₑₓ is higher than in the absence of HCO₃⁻, the [H₄CO₃]₀ is also higher, approximately 24 mM. The calculated buffering capacity is therefore approximately 84 H⁺/pH unit. This is consistent with the addition of acetate and HCO₃⁻.
**Intracellular pH of 3T3 Cells**

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**FIG. 1.** Response of pH<sub>i</sub> of BALB/c 3T3 cells as measured by the fluorescence of pyranine. Cells were prepared as described under "Materials and Methods." Fluorescence was monitored at 465, 415, and 405 nm excitations using an SLM 4800 scanning fluorometer. In A–E cells were incubated either in the absence (A) or the presence (B–E) of physiological concentrations of HCO<sub>3</sub>·. Then challenged with isosmotic CSB containing 50 mM acetate in lieu of NaCl. F–I, cells were perfused with HCO<sub>3</sub>-free CSB and challenged with HCO<sub>3</sub>-containing CSB. In J and 2 cells were perfused with either HCO<sub>3</sub>-free CSB (I) or complete CSB (J), then challenged with HCO<sub>3</sub>-depleted CSB. In all experiments, pH<sub>i</sub> values were confirmed by perfusing cells with nigericin/K<sup>+</sup> buffer, pH 7.05, at the end of each experiment (15).

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**TABLE 1**

<table>
<thead>
<tr>
<th>Initial condition</th>
<th>Treatment</th>
<th>dpH&lt;sub&gt;i&lt;/sub&gt;/dt&lt;sup&gt;max&lt;/sup&gt;</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>pH&lt;sub&gt;i&lt;/sub&gt;&lt;sup&gt;min&lt;/sup&gt;</th>
<th>pH&lt;sub&gt;i&lt;/sub&gt;&lt;sup&gt;max&lt;/sup&gt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) - HCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>50 mM NaOAc</td>
<td>7.2 ± 2.7</td>
<td>33.8 ± 8.5</td>
<td>7.14 ± 0.03</td>
<td>6.90 ± 0.08</td>
<td>6</td>
</tr>
<tr>
<td>(b) + HCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>50 mM NaOAc</td>
<td>13.2 ± 2.5</td>
<td>10.4 ± 2.7</td>
<td>7.15 ± 0.03</td>
<td>6.90 ± 0.08</td>
<td>6</td>
</tr>
<tr>
<td>(c) - HCO&lt;sub&gt;3&lt;/sub&gt; + DIDS</td>
<td>50 mM NaOAc</td>
<td>8.9 ± 1.3</td>
<td>51.3 ± 15.0</td>
<td>7.16 ± 0.04</td>
<td>6.92 ± 0.27</td>
<td>3</td>
</tr>
<tr>
<td>(d) - HCO&lt;sub&gt;3&lt;/sub&gt; + CI</td>
<td>50 mM NaOAc</td>
<td>5.3 ± 1.2</td>
<td>52.8 ± 12.7</td>
<td>7.15 ± 0.04</td>
<td>7.08 ± 0.09</td>
<td>3</td>
</tr>
<tr>
<td>(e) - HCO&lt;sub&gt;3&lt;/sub&gt; + Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>50 mM NaOAc</td>
<td>6.6 ± 0.8</td>
<td>56.8 ± 15.0</td>
<td>7.16 ± 0.06</td>
<td>7.08 ± 0.09</td>
<td>3</td>
</tr>
<tr>
<td>(f) - HCO&lt;sub&gt;3&lt;/sub&gt; + DIDS</td>
<td>24 mM NaOAc</td>
<td>23.0 ± 1.1</td>
<td>6.5 ± 0.8</td>
<td>7.11 ± 0.03</td>
<td>6.90 ± 0.18</td>
<td>3</td>
</tr>
<tr>
<td>(g) - HCO&lt;sub&gt;3&lt;/sub&gt; + CI</td>
<td>24 mM NaOAc</td>
<td>30.0 ± 2.4</td>
<td>21.7 ± 7.6</td>
<td>7.10 ± 0.01</td>
<td>6.93 ± 0.10</td>
<td>3</td>
</tr>
<tr>
<td>(h) - HCO&lt;sub&gt;3&lt;/sub&gt; + Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>24 mM NaOAc</td>
<td>22.0 ± 0.8</td>
<td>6.5 ± 0.8</td>
<td>7.12 ± 0.03</td>
<td>6.93 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>(i) - HCO&lt;sub&gt;3&lt;/sub&gt; + DIDS</td>
<td>10 mM NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>15.7 ± 0.7</td>
<td>12.3 ± 0.7</td>
<td>7.19 ± 0.04</td>
<td>7.16 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>(j) - HCO&lt;sub&gt;3&lt;/sub&gt; + Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10 mM NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>7.5 ± 0.2</td>
<td>12.3 ± 0.7</td>
<td>7.09 ± 0.08</td>
<td>7.12 ± 0.12</td>
<td>4</td>
</tr>
</tbody>
</table>

* Velocity of pH<sub>i</sub> recovery (× 10<sup>-3</sup> pH unit·min<sup>-1</sup>) calculated at pH<sub>i</sub> in 6.80.
* Time to half-maximal pH change (minutes).
* External pH ± S.D. throughout experiment, as measured in the fluorometer cuvette.
Intracellular pH of 3T3 Cells

H, and Table I, h and i. As shown in the figures, these treatments also increased the magnitude and lifetime of the transient acidification associated with the addition of HCO₃⁻. Notice that Na⁺ removal does not completely inhibit the rate of HCO₃⁻-induced alkalization. This indicates the presence of a Na⁺-independent transport activity, possibly involving transbilayer movement of H⁺ equivalents.

The Intracellular pH Change Induced by Addition of HCO₃⁻ Is Maximal at Low Concentrations—The increases in pHₐ observed in Fig. 1, F–H, and Table I, g–i, were induced by the addition of 24 mM HCO₃⁻ to cells that were previously in the absence of HCO₃⁻. This effect of HCO₃⁻ on the pHₐ of HCO₃⁻-depleted cells is concentration-dependent, as shown in Fig. 2A. In this experiment, perfusion was begun in 0.1 mM HCO₃⁻ and, at various times thereafter, pH = 7.00 buffer of increasing concentrations of HCO₃⁻ were added, up to a maximum of 25 mM. Under these conditions, each increase in HCO₃⁻ concentration induces a transient acidification followed by a prolonged alkalization to a new steady-state pHₐ. Cytoplasmic alkalization is virtually complete after the addition of HCO₃⁻ to 5 mM. Fig. 2B illustrates data from several such experiments, wherein the pHₐ recovery was determined at various concentrations of HCO₃⁻. These data show that half-maximal alkalization is achieved at 0.05 mM HCO₃⁻.

Recidification of the Cytoysm in Response to an Alkaline Load Occurs with Slow Kinetics—The bidirectionality of HCO₃⁻ transport was investigated by monitoring the recovery of cells to an ammonium-induced alkaline load. In these experiments, cells were first perfused with CSB (with or without HCO₃⁻), followed by perfusion with CSB containing NH₄Cl, which induces a rapid alkalization, followed by a recidification. As shown in Fig. 1, I and J, and Table I, j and k, the kinetics of recidification are slightly faster in the presence of HCO₃⁻. The pHₐ recovery rates reported here are slower than those reported elsewhere (for reviews, see Refs. 1–4). Notice also that, in the absence of HCO₃⁻, NH₄Cl induces a slower alkalization, to a lower final pHₐ, as compared to cells in the presence of HCO₃⁻.

HCO₃⁻ Attenuates the pHₐ Response to Nu-Serum in a pHₐ-dependent Fashion—in Fig. 3, A–C, we illustrate the effect of Nu-Serum on the pHₐ of BALB/c 3T3 cells under three different conditions: high pHₐ with HCO₃⁻ (Fig. 3A), low pHₐ with HCO₃⁻ (Fig. 3B), and low pHₐ, without HCO₃⁻ (Fig. 3C). In the cells without HCO₃⁻, the pHₐ response is large; increasing by 0.4 pH unit over a period of 30 min. At the same pHₐ in the presence of HCO₃⁻, the resting pHₐ is higher than in the absence of HCO₃⁻; and Nu-serum induces an increase in pHₐ, to approximately the same value observed in HCO₃⁻-free buffer. In the presence of HCO₃⁻ at high pHₐ, Nu-serum not only does not raise the pHₐ, it causes a decrease in pHₐ. Fig. 4A illustrates the entire titration curve for these phenomena. In this figure, we present the data as the change in pHₐ caused by serum, as a function of the external pH in the presence and absence of HCO₃⁻. Note that, at high pHₐ values (7.8), the two curves virtually coincide, indicating no effect of HCO₃⁻ on the pHₐ response. At moderate pHₐ values (7.0–7.5), the pHₐ response of cells in the presence of HCO₃⁻ is attenuated relative to that of cells in the absence of HCO₃⁻. Also, notice that, in the presence of HCO₃⁻, there is no increase in pHₐ at pHₐ values above 7.5. Fixating the change in pHₐ as a function of pHₐ yields the curves shown in Fig. 4C. From pHₐ 6.7 to 7.6, the effect of Nu-serum on pHₐ is dependent only on the initial value of pHₐ and is not affected by the presence or absence of HCO₃⁻.

HCO₃⁻ Attenuates Activation of Na⁺/H⁺ Exchange by Nu-Serum at High pHₐ—The previous experiments indicate that HCO₃⁻ raises pHₐ and attenuates serum-induced changes in pHₐ. However, they do not directly demonstrate the involvement of Na⁺/H⁺ exchange in this process. To investigate this, we have monitored the effect of Nu-Serum on amiloride-inhibitable Na⁺ influx in the presence of physiological HCO₃⁻ levels. These results are shown in Fig. 4B. In these experiments, cells are incubated at the various pHₐ values in the presence and absence of ouabain, amiloride, and/or Nu-serum. In the absence of ouabain, the Na⁺ levels are steady-state in the presence or absence of amiloride and/or Nu-serum (data not shown). Addition of ouabain prevents the extrusion of Na⁺ and, hence, the rate of increase in intracellular Na⁺ is a measure of its inward-directed flux (17). In these experiments, we took samples at various times after the addition of ouabain in the presence and absence of Nu-serum and/or amiloride, fit the data by least squares linear regression, and took the slope (dNa⁺/dt) as the flux. The difference between exchange activity in the presence and absence of Nu-serum (dNa⁺/dt) is the rate of serum-stimulable Na⁺ influx. As shown in Fig. 4B, this rate increases with decreasing pH, reaching a maximum at pHₐ = 6.8. This influx is abol-

![Fig. 2. Response of BALB/c 3T3 cells pH to addition of HCO₃⁻ as measured by fluorescence of pyranine. Conditions for A were identical to those described for Fig. 1F, except that HCO₃⁻ concentration in buffer was increased stepwise. B represents the mean ± S.D. from 5 similar experiments. Dotted line represents least-squares fit to one-component exponential. This fit indicates a half-maximal alkalization at 0.05 mM.](image)

![Fig. 3. Response of cells to the addition of 10% Nu-serum at different pHₐ values in the presence (A and B) and absence (C of HCO₃⁻) as measured by pyranine. Cells were perfused under the conditions indicated in the presence of 0.2% Nu-serum. At the times indicated by the arrows, perfusate was switched for one containing 10% Nu-serum.](image)
ished with amiloride. The data used to generate this Figure indicate that the effect of amiloride is pH-dependent (e.g. increasing with decreasing pH) in the presence or absence of serum. We take these observations to suggest that amiloride acts by inhibiting Na/H exchange. As illustrated in this Figure, there is no effect of Na-serum on Na influx at a pH above 7.4, yet there are significant effects at pH values between 7.0 and 7.3. Notice that the effect of amiloride on serum-stimulated Na influx coincides with its effects on serum-induced changes in pH. (cf. Fig. 4, A and B). In parallel experiments we observed that pH was approximately equal to pH., under these conditions. Therefore, transformation of data from pH, to pH. involved only minor corrections. Fig. 4D illustrates the effect of varying pH on the serum-stimulated, amiloride-inhibited Na influx. This curve is very similar in appearance to that shown in Fig. 4C, suggesting that the change in pH, and Na influx are related (e.g. via Na+/H+ exchange).

**DISCUSSION**

**Anion Exchanger of BALB/c 3T3 Cells Is Sodium-dependent Cl-/HCO3 Exchange**—The accumulated data in this study indicate that the HCO3 transport system of BALB/c 3T3 cells appears to be a member of the NaHCO3/HCl exchange family. Fig. 1, A and B, and Table I, a and b, illustrate that recovery of the cytoplasm from an acetate-induced acid load is significantly stimulated by the presence of HCO3. This HCO3-stimulated recovery is inhibited by DIDS and requires the presence of both Na and Cl (Fig. 1, C, D, and E, and Table I, a, d, and f). HCO3-stimulated recovery is not affected by amiloride. Data in the presence of HCO3 and 1-10 mM amiloride are indistinguishable from those in Fig. 1B and Table 1B. Data in the absence of HCO3 and presence of serum and 10 mM amiloride are identical to those in Fig. 1A. The data in Fig. 1, P-H, and Table I, a, illustrate that HCO3 entry into these 3T3 cells increases the pH, and this increase is also inhibited by DIDS and requires external Na+. These data support the conclusion that HCO3 buffering in BALB/c 3T3 cells utilizes Na-dependent Cl-/HCO3 exchange. Since the current data do not indicate the stoichiometry of the transported species, we cannot unequivocally state the exact nature of the transporting activity, or even whether it is electrogenic.

**HCO3 Buffering System in BALB/c 3T3 Cells Is Dynamic**—The effect of HCO3 on the pH, is maximal above a concentration of 5 mM, as shown in Fig. 2. These data indicate that addition of as little as 0.5 mM HCO3 can cause a significant intracellular alkalization. For 0.55 mM HCO3 to have a measurable effect on pH, we believe that the HCO3 buffering system in these cells must involve a rapid recycling of CO2 across the membrane. The physiological relevance of these rapid kinetics is impossible to assess without reliable data on the magnitude of intracellular acid load.

Under conditions where the HCO3 transport system is inhibited and only basal activity of Na+/H exchange is present, the recovery from an acid load occurs at a Vmax of 8 x 10^-5 pH unit/min (Table Ia). This should be contrasted to the recovery rate of 35 x 10^-4 pH unit/min in the presence of active NaHCO3/HCl exchange (Table Ib). This comparison indicates that HCO3 buffering is significantly more active than is unstimulated Na+/H exchange.

**HCO3 Attenuates Response to Nu-Serum in a pH-dependent Fashion**—The above data consistently indicate that HCO3 raises the pH, of BALB/c 3T3 cells. Figs. 3 and 4 illustrate that HCO3 and/or high pH, can raise the pH, to a value high enough to preclude further alkalination induced by mitogens. The pH, value at which there is no effect of Nu-serum is lower in the presence of HCO3 (7.3) than in its absence (7.5). These values are interesting, because a pH, of 7.3 is the presence of physiological HCO3 are typical for culture conditions. These observations are consistent with the literature in that, some researchers have observed no pH, response to Nu-serum or growth factor addition in the presence of HCO3, whereas others have (see Refs. 1 and 2 for reviews). Since most of these studies do not systematically report the pH, values, the most likely explanation for this discrepancy is that those observing a pH, change were working at a lower pH,. The data in the range 7.0-7.3 illustrate that HCO3 attenuates, but does not necessarily prevent, the pH, response. Under more acidic pH, conditions, the pH, response of cells in the absence of HCO3 is not observed, whereas it is enhanced in the presence of HCO3. This is probably due to the inhibition of the Na+/H exchange at more acidic pH, values (21). Since HCO3 maintains a higher pH, the exchanger is not inhibited at these acidic pH, conditions. In all of these studies, we have used HCO3 at physiological concentrations. This means that, as the pH, is lowered, the HCO3 concentration decreases, such that, at
**Intracellular pH of 3T3 Cells**

pH₄, 6.8, the bicarbonate concentration is only 6 mM. However, HCO₃⁻-dependent pH₄ regulation is very dynamic, with only 0.5 mM required to effect a significant increase in pH₄ (see above).

**Na⁺/H⁺ Exchange Is Not Activated under Culture Conditions**—The previous data show that the pH₄ response of 3T3 cells to Nu-serum is complex, but that it follows a pattern. A model for this phenomenon includes cellular alkalinization caused by HCO₃⁻ and/or high pH₄. If pH₄ is above the activated set point of the Na⁺/H⁺ exchanger, there will be no pH₄ response to mitogens. However, the previous data do not address the involvement of the Na⁺/H⁺ exchanger. In the experiments illustrated in Fig. 4, B and D, we addressed the involvement of the Na⁺/H⁺ exchanger directly by monitoring the effect of Nu-serum on the rate of amiloride-inhibitable Na⁺ entry. These data indicate that Na⁺/H⁺ exchange is not activated at pH₄ values above 7.3 in the presence of physiological HCO₃⁻ concentrations. This pH₄ is similar to the values at which there is no effect of Nu-serum on the pH₄ (cf., Fig. 4, A and C), suggesting that these phenomena are coupled. This is substantiated when the data from Figs. 4, A and C, are plotted as a function of pH₄. Fig. 4C shows that the magnitude of mitogen-induced alkalinization is dependent solely on the initial pH₄. Comparison of Fig. 4C and D, illustrates that the pH₄ response in the presence of HCO₃⁻ closely parallels the response of Na⁺/H⁺ exchange. The effect of HCO₃⁻ in attenuating the response of Na⁺/H⁺ exchange is therefore predictable by its effects on the pH₄.

Although the data in the present experiments illustrate a requirement of HCO₃⁻ transport for both Cl⁻ and Na⁺, it does not readily resolve the stoichiometry of HCO₃⁻/Cl⁻/Na⁺. Regardless of the exact identity of the HCO₃⁻ exchanger, it is extremely active under physiological conditions. To illustrate this, we observed that as little as 0.55 mM HCO₃⁻ can cause a significant intracellular alkalinization.

The above data also indicate that HCO₃⁻ attenuates the pH₄ response of cells to Nu-serum to a degree consistent with its effects on pH₄. Under typical culture conditions (pH₄ 7.2-7.4, physiological HCO₃⁻), mitogenic stimulation would not produce a cytoplasmic alkalinization. Therefore, it appears that in vitro, the pH₄ response of cells to mitogens cannot be a stimulus of proliferation per se. Why, then, have virtually all cells evolved this, we observed that as little as 0.55 mM HCO₃⁻ can cause a significant intracellular alkalinization.

In summary, we have described a HCO₃⁻-dependent pH₄ regulating system in BALB/c 3T3 cells which acts in a very dynamic fashion to raise, but not lower, the pH₄ under physiological conditions. This system is driven by gradients for HCO₃⁻, H⁺, Cl⁻, and Na⁺. The data in this study suggest that, at external pH values between 7.0 and 7.2, this exchanger acts to set pH₄ equal to pH₆. We have also defined the boundary conditions for the mitogenic stimulation of cytoplasmic alkalinization under physiological (HCO₃⁻-containing) conditions. Our findings indicate that Na⁺/H⁺ exchange and, hence pH₄, is not affected by mitogenic stimulation under culture conditions. However, pH₄ may be indirectly affected in culture by the increased buffering capacity caused by modification of the Na⁺/H⁺ exchanger. It is also possible that pH₄ alkalinizes in response to mitogens in situ, and that this activity may have physiological significance.

Acknowledgments—We would like to thank G. M. Martin for her technical assistance in all phases of this study. We would especially like to thank K. A. Giuliano (Carnegie-Mellon University), Steve Wright (University of Arizona), and J. W. Nichols (Emory University) for their helpful discussions and criticisms during all phases of this project.

REFERENCES


The above data also indicate that the pH₄ response of cells to Nu-serum to a degree consistent with its effects on pH₄. Under typical culture conditions (pH₄ 7.2-7.4, physiological HCO₃⁻), mitogenic stimulation would not be expected to produce a cytoplasmic alkalinization. Therefore, it appears that in vitro, the pH₄ response of cells to mitogens cannot be a stimulus of proliferation per se. Why, then, have virtually all cells evolved this mechanism of stimulating Na⁺/H⁺ exchange upon mitogenic activation? There are two possible answers which are not mutually exclusive: 1) that stimulation of Na⁺/H⁺ exchange increases the apparent active buffering capacity of cells in anticipation of a metabolic acid load, or that 2) these cells evolved in tissues, which have lower pH₄ environments than those observed in vitro. In the first case, mitogenic stimulation would not produce a direct alkalization, but would do so indirectly by preventing an acidification. Indeed, pH₄ decreases upon amiloride treatment or in response to Nu-serum at high (≥7.5) pH₄ values (data submitted for review). In support of the second hypothesis, the pH₄ is in the range of 6.8-7.0 as measured using a variety of techniques (22). Under these conditions, mitogenic stimulation would be expected to produce an alkalinization, which might therefore be a trigger in situ. This hypothesis is emphasized by the recent findings of Perona and Serrano (23), who showed that transfection of 3T3 cells with a yeast H⁺-ATPase induced an increase in pH₄ and caused these normal cells to become tumorigenic.
APPENDIX II.

POSSIBLE INVOLVEMENT OF H⁺-ATPases IN MAMMALIAN CELL TUMORIGENICITY.
SECTION 1.

Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions.

Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions

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Communicated by Efraim Racker, June 16, 1990 (received for review April 4, 1990)

ABSTRACT One of the earliest events in the response of mammalian cells to mitogens is activation of Na⁺/H⁺ exchange, which increases intracellular pH (pHᵢ) in the absence of HCO₃⁻ or at external pH values below 7.2. The proliferative response can be blocked by preventing the pHᵢ increase; yet, the proliferative response cannot be stimulated by artificially raising pHᵢ with weak bases or high medium pH. These observations support the hypothesis that optimal pHᵢ is a necessary, but not sufficient, component of the proliferative-response sequence. This hypothesis has recently been challenged by the observation that transfection of NIH 3T3 cells with yeast H⁺·ATPase renders them tumorigenic. Although previous measurements indicated that these transfected cells maintain a higher pHᵢ in the absence of HCO₃⁻, whether H⁺·ATPase transfection raised the pHᵢ under physiologically relevant conditions was not known. The current report shows that these transfected cells do maintain a higher pHᵢ than control cells in the presence of HCO₃⁻, supporting the possibility that elevated pHᵢ is a proliferative trigger in vivo. We also show that these cells are serum-independent for growth and that they glycolyze much more rapidly than phenotypically normal cells.

All mammalian cells maintain a higher intracellular pH (pHᵢ) than predicted from Nernst equilibrium (1). Maintenance of this pH gradient can be mediated by three transport systems: Na⁺/H⁺ exchange, HCO₃⁻ transport, and plasma membrane H⁺·ATPase. Most, if not all, cells contain Na⁺/H⁺ exchange and HCO₃⁻ transport activities, whereas plasma membrane H⁺·ATPase activity is generally observed only in specialized epithelia, such as bladder, intestine, and kidney (2). Interplay between these two (and sometimes three) systems buffers the pHᵢ against changes in the extracellular pH (pHₑ) and can mediate changes in pHᵢ during certain physiological responses.

One of the earliest events in the response of mammalian cells to mitogens is activation of Na⁺/H⁺ exchange (3, 4). In the absence of HCO₃⁻ or at low pHᵢ, this increases pHᵢ (5, 6). Although it was earlier thought that elevated pHᵢ might be a second messenger in the proliferative response (7, 8), current hypotheses regard elevated pHᵢ as only permissive for proliferation (4, 5). To assign a causal role for pHᵢ in proliferation, elevated pHᵢ must be seen upon stimulation, inhibition of the increase in pHᵢ must inhibit proliferation, and mimicking the increase must be sufficient to stimulate proliferation. These conditions are not satisfied in vitro. Although preventing the increase in pHᵢ generally inhibits proliferation (3, 4, 9), many cells do not increase their pHᵢ high at pHᵢ (e.g., pH 7.4) in the presence of bicarbonate (5, 6, 10, 11), and artificially raising pHᵢ with weak bases or high pHᵢ is not sufficient to stimulate proliferation (12, 13). It is possible, however, that these conditions are not satisfied in vivo. For instance, we and others have shown that mammalian cells will alkalize upon mitogenic stimulation under conditions expected in vivo (e.g., pH 7.0–7.2) (cf. refs. 5, 6, and 14).

Additionally, Perona and Serrano have demonstrated that in vivo tumorigenicity can be induced in phenotypically normal NIH 3T3 mouse embryo cells by transfection with yeast H⁺·ATPase (15). These cells, clone in soft agar, grow to higher density, and are tumorigenic in nude mice. There is a number of consequences of this transfection which may be important to the tumorigenic response—such as elevated pHᵢ, increased membrane potential (16), or increased rate of ATP turnover. Although the activity of the H⁺·ATPase could be expected to increase pHᵢ in vivo, there are two other H⁺-transporting systems in these cells (e.g., Na⁺/H⁺ exchange; Na⁺·dependent Cl⁻/HCO₃⁻ exchange) the activity of which could override effects of the H⁺·ATPase. A fundamentally important unsolved question in this system is whether the pHᵢ of these tumorigenic cells is higher under physiologically relevant conditions.

In the present communication, we accurately measure the pHᵢ of these cells vis-a-vis normal NIH 3T3 cells and cells transfected with an inactive form of the H⁺·ATPase and report that the pHᵢ of the tumorigenic cells is significantly more alkaline than that of the normal cells under physiological conditions. We have also examined the effects that alteration of pHᵢ may have on cellular physiology that is permeable to the transformed phenotype. These tumorigenic cells do not grow faster than their normal counterparts under normal culture conditions. However, they do grow to much higher densities and have lost their serum requirement for growth. We have also observed that these cells exhibit a rapid rate of aerobic glycolysis and that this increased activity cannot be accounted for by the elevated pHᵢ alone. The cause of tumorigenicity in these cells could lie in their altered regulation of pHᵢ, which may manifest itself through serum-independence, and elevated pHᵢ may be a proliferative trigger in vivo.

MATERIALS AND METHODS

Cells. R11 cells were produced as described (15, 16). Briefly, 300,000 NIH 3T3 cells were transfected using 0.1 µg of pSVα-rDNA plasmid, 8 µg of carrier DNA, and 0.5 µg of pSVα-AT plasmid, which contained the gene for the yeast plasma membrane H⁺-ATPase (allelic PMA1) under control of the simian virus 40 promoter. Clones were selected with G-418

Abbreviations: pHᵢ, intracellular pH; pHₑ, extracellular pH; SNARE-F1, N,N-dimethyl-1,15-hexaboyseminophosphorodihaloacetate; BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; CSB, cell suspension buffer; HBSS, Hanks' balanced salt solution.

1To whom reprint requests should be addressed.
and isolated by cylinder cloning. N-mut cells were prepared in the same way but contain allele pma213: a Glu223 → Gln mutation that gives rise to an “uncoupled” form of the H⁺-ATPase, with 40% of the ATPase activity and 20% of the H⁺-pumping activity as compared with PMAF (16, 17). BALB/c 3T3 mouse embryo fibroblasts were obtained from American Type Culture Collection (ATCC CCL 153). NIH 3T3 cells were gifts from Gene DuChes (Colorado State University, Fort Collins, CO). Although NIH 3T3 cells were the parent line for all transfectants, we also used BALB/c 3T3 cells as controls in this study because they are much better characterized. Previous studies in our lab have shown no differences in the pH^* regulation between NIH and BALB/c cells (18).

A number of lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GibCO), supplemented with 10% Nu-Serum (Collaborative Research). The initial inocula as received from the culture center grew to a density of 70% confluence in 300 cm² at which time the cells were frozen in DMEM/10% dimethyl sulfoxide/20% Nu-Serum at a density of 1 × 10⁶ cells per freezing ampule. Cells from these frozen stocks were recovered every 6 weeks in 75-cm² tissue culture flasks and passed bi-weekly at a density of 2 × 10⁶ cells per 75-cm² flask. Cells were subcultured for 2 weeks before use in experiments.

For fluorescence experiments, cells were plated into Petri dishes containing 9 × 22 mm coverslips at a density of 10⁶ cells per dish in DMEM/10% Nu-Serum. On the following day, these cultures were prepared for fluorescence determination of pH^* as described below. For other experiments, cells were consistently plated at a density of 5 × 10⁶ cells per cm².

Chemicals and Buffers. All dyes were purchased from Molecular Probes. Amiloride, nigericin, and valinomycin were all purchased from Sigma.

Cell Suspension Buffer (CSB). CSB contained 1.3 mM CaCl₂/1 mM MgSO₄/5.4 mM KCl/0.44 mM KH₄PO₄/110 mM NaCl/0.35 mM Na₂HPO₄/24 mM NaHCO₃/5 mM glucose/2 mM glutamine. The pH of CSB and media was maintained with 50 mM Mes/Hepes/Tricine. For HCO₃⁻-free CSB, 24 mM NaCl was substituted for the NaHCO₃ in CSB. Hanks’ balanced salt solution (HBSS) was 138 mM NaCl/10 mM CaCl₂/1 mM MgCl₂/5.4 mM KCl/0.35 mM Na₂HPO₄/5 mM glucose/2 mM glutamine/0.03 Mm phenol red. The salt composition of all buffers was prepared at least a day before experiments to allow for equilibrium hydration of CO₂, whereas the organic components were always added immediately before experiments to avoid bacterial contamination.

Measurement of pH^*. Intracellular pH was determined by fluorescence of either N,N-dimethyl-1,5,6-bis(carboxysemide)naftphorhodolplor SNARF-1 or 2,7'-bis(2-carboxyethyl)-5-carboxyfluorescein (BCECF). The dyes were loaded into cells grown on coverslips in their acetoxymethyl ester forms, as described (19). Briefly, coverslips were incubated for 30 min at 37°C in CSB containing 0.2 μM SNARF-1 or 2 μM BCECF in their respective acetoxymethyl ester forms. After this, cells were rinsed three times with CSB and incubated 30 more min to ensure complete ester hydrolysis. Fluorescence was determined with an SLM-8000C spectrophotometer outfitted with a flowthrough device for sample perfusion (20). Two coverslips containing cells were mounted in the holder back to back and were perfused at 1 ml/min. Sample temperature was maintained at 37°C by keeping both the water jacket and the perfusion medium at 37°C. The fluorescence-ratio methods used for these dyes are described elsewhere (18, 19, 21).

Measurement of Acid Production. Cells were plated into 96-well microtiter plates (Flow Laboratories) and allowed to grow to complete confluency (~2 days). At the time of experiment, medium was aspirated and wells were washed three times with unbuffered HBSS. Cells were then incubated in 100 μl of unbuffered HBSS, and the absorbance of phenol red was monitored at 450 nm and 490 nm for 30 min by using a microtitre plate reader (Bio-Tek, Burlington, VT). The ratio of 450/490 absorbance can be converted to pH by using the equation:

\[ pH = pK_a + \log \left( \frac{R - R_{min}}{R_{max} - R} \right) \]  

where R is the measured ratio of absorbance at two wave lengths, Rmax is the ratio of the anion form of dye, and Rmin is the ratio of the protonated dye. In the case of phenol red, pKₐ = 7.50, Rmin = 0.2, and Rmax = 2.5. The dpH/dt was calculated and converted to H⁺ equivalents by correcting for buffering capacities, which were 330 nM H⁺ per pH unit for amiloride-containing solutions and 147 μM H⁺ per pH unit for amiloride-free solutions, as determined by titration of stock solutions.

Previously Described Analyses. Glucose and lactate concentrations in media were monitored with an automated, simultaneous glucose/lactate analyzer (Yellow Springs Instruments). H⁺-ATPase protein was measured by immunofluorescence, and the presence of H⁺-ATPase DNA was monitored by Southern analysis (15). Cell mass was determined by crystal violet staining and converted to cell number, as described (22). Intracellular K⁺ was determined by atomic absorption spectroscopy (23). Least-squares analysis and simplex curve fitting were done with MINOS (MicroMath Software).

RESULTS

Dye Calibration. Measurement of absolute pH^* for comparison between different cell types is not simple, although the process is straightforward. The most sensitive method for measuring pH^* in mammalian cells uses fluorescence ratios (21). However, dye characteristics can be affected by the composition of the internal milieu (24). To correct for potential artifacts, the behavior of each dye must be accurately determined in situ for each cell type examined. Examples of these data are illustrated in Fig. 1 and 2, where we express the pH dependence of fluorescence ratios for two dyes: SNARF-1 and BCECF in three types of cells: NIH3T3 cells transfected with wild-type allele, N-mut cells transfected with mutant allele pma213, and normal BALB/c 3T3 cells. The pH gradients of these cells have been collapsed by using a combination of 147 mM KCl, 2 mM valinomycin, and 6.8 mM nigericin. The high K⁺ is used to approximate intracellular K⁺ concentration, as determined by atomic absorption. Valinomycin completes the collapse of the K⁺ gradient without significant effects on cell volume, and nigericin sets the H⁺ gradient equal to the K⁺ gradient, which, in this case, is unity. As illustrated, the ratios of both SNARF-1 and BCECF are sensitive to pH. Ratios at values >pH 8.0 are not obtainable, as discussed (19). By using least-squares analysis, these data are iteratively solved for pKₐ, Rmin, and Rmax in Eq. 1. As shown in Table 1, these values slightly differ between the different cell types (see below).

Intracellular pH. The effects of external pH (pH^*) on the pH^* of these cells are illustrated in Fig. 2. These data were obtained by perfusing cells with CSB containing HCO₃⁻ at concentrations set by equilibrium with 5% CO₂ atmosphere (see Fig. 2 legend). After each determination, cells were perfused with a buffer containing nigericin, valinomycin, and high K⁺ at pH 7.2 to obtain a calibration point. These calibrations were used to correct the observed ratios with the titration curves in Fig. 1. This procedure corrects for minor differences in dye loading between samples (19). In practice, these correction factors were consistently insignif-
Fig. 1. Fluorescence ratio of intracellularly loaded SNARF-1 and BCECF in 3T3 cells, the pH gradient of which has been collapsed. RN1a, N-mut, and BALB/c 3T3 cells were prepared and loaded with dyes, as described. Cells were perfused with buffer containing 147 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 2 μM valinomycin, and 6.8 μM nigericin. Data were collected with an SLM 8000c spectrophotometer and are expressed as the fluorescence ratio produced at excitation wavelengths of 500 nm and 450 nm for BCECF (λmax = 529 nm) and the fluorescence ratio observed at emission wavelengths of 644 nm and 584 nm for SNARF-1 (λmax = 534 nm).

Buffer pH was determined with a Beckman pH meter and a gel-filled combination glass electrode calibrated at 37°C to pH 7.0 and 10.0 with commercially available solutions (VWR). Solid, dashed, and dotted lines represent data fit to Eq. 1 from data in Table 1 for RN1a, N-mut, and 3T3 cells, respectively.

Propagating these values into the data of Fig. 2 eliminates the significance between N-mut and 3T3 cells; yet, both remain significantly different from RN1a cells to a 95% confidence interval. Therefore, by using two dyes and correcting for possible artifacts and propagating errors, we can state with high confidence that the pH of tumorigenic RN1a cells is significantly higher than that of their nontumorigenic counterparts. A remaining problem is the difference in the absolute pH determined with the two dyes. Although both dyes consistently show that the pH of RN1a cells is higher than that of the phenotypically normal cells, SNARF-1 consistently reports a higher pH relative to that obtained with BCECF. The cause for this discrepancy between the dyes is unknown.

Cell Growth and Serum Requirement. Because the pH of the RN1a cells is higher than that of 3T3 cells, we might also predict that they would grow at a faster rate because pH

Table 1. Parameters for estimating pH by using SNARF-1 and BCECF in situ

<table>
<thead>
<tr>
<th>Cell type</th>
<th>pH</th>
<th>Rmin (nm)</th>
<th>Rmax (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>7.39 ± 0.110</td>
<td>0.422 ± 0.016</td>
<td>1.837 ± 0.170</td>
</tr>
<tr>
<td>N-Mut</td>
<td>7.39 ± 0.131</td>
<td>0.456 ± 0.008</td>
<td>1.482 ± 0.150</td>
</tr>
<tr>
<td>RN1a</td>
<td>7.338 ± 0.156</td>
<td>0.310 ± 0.024</td>
<td>1.908 ± 0.307</td>
</tr>
<tr>
<td>BALB/c</td>
<td>6.936 ± 0.025</td>
<td>1.681 ± 0.042</td>
<td>6.884 ± 0.077</td>
</tr>
<tr>
<td>RN1a</td>
<td>6.967 ± 0.061</td>
<td>0.999 ± 0.088</td>
<td>6.509 ± 0.195</td>
</tr>
</tbody>
</table>

Numbers in boldface represent median values. Statistical analysis was done by using Student’s t test; unreported differences are not significant.

*P < 0.001 N-mut versus BALB/c.

**P < 0.005 N-mut versus BALB/c or RN1a.

***P < 0.0005 RN1a versus BALB/c cells.
Correlates with growth rate in a number of cell types (25): this is not observed. Fig. 3A illustrates that, at low density, 3T3 cells grow faster than RN1a cells at pH values below 6.8. Although they grow slower, RN1a cells grow to much higher densities than do 3T3 cells, as evidenced by cell number after 9 days in culture (Fig. 3B). The data from higher-density cultures compare favorably with those obtained recently by Feron et al. (16).

Significantly, RN1a cells are not dependent upon serum supplementation for growth (Fig. 3C). The pH measurement shown in Fig. 2 were made without serum, and the growth studies of Fig. 3A and B were done with 10% Nu-Serum. Nu-Serum addition raises the pH of 3T3 cells to be similar to that of the RN1a cells at neutral pH (6), illustrated by the dotted line in Fig. 3A. Therefore, with serum, we would expect the pH differences to be less significant.

**DISCUSSION**

Is altered pH regulation related to tumorigenesis? Clearly, in this artificial construct, the H⁺-ATPase-transfected, tumorigenic RN1a cells have a higher pH under physiological (e.g., HCO₃⁻-containing) conditions than do their nontumorigenic counterparts. Although there is no change in the pH of normal cells in response to mitogens under culture conditions (e.g., pH 7.3-7.4), there are significant increases in pH at the lower pH values expected in vivo (e.g., 7.0-7.2) (5, 6, 15). These tumorigenic cells, therefore, have a pH without serum that is higher than that of normal cells with serum. Significantly, these tumor cells are also serum-independent for growth, which raises the possibility that the
supraphysiological pH\textsuperscript{x} circumspect other mitogen-induced events and stimulates growth directly.

Alkalization might also be involved in natural tumorigenesis. Growth cessation in vivo may be induced by absence of growth factors or density-dependent inhibition. These data are consistent with a model wherein a physiological signal for growth cessation is lost of pH\textsuperscript{x} regulation and, hence, intracellular acidification. H\textsuperscript{+}-ATPases may be refractory to such regulation, allowing otherwise inhibited cells to maintain a high pH\textsuperscript{x} and, therefore, grow.

Could H\textsuperscript{+}-ATPases be involved in natural carcinogenesis? There is evidence for an electrogenic H\textsuperscript{+}- exporting ATPase in Ehrlich ascites tumor (EAT) cells (26), enhanced H\textsuperscript{+}- extruding activity is expressed upon transformation of Chinese hamster ovary cells (27), and ATP is directly required for pH\textsuperscript{x} regulation in both Aα-31 tumor cells (28) and EAT cells (29). Plasmaememal H\textsuperscript{+}-ATPase expressed in the non-pnephel cells might be a plasma membrane type H\textsuperscript{+}/K\textsuperscript{+}- ATPase, or it could be a vacular-type, as suggested by Racker (30). Pathological expression of a chitin-coated vesicle or vacular H\textsuperscript{+}-ATPase could arise from something as simple as increased retention times in the plasma membrane during recycling. Vacular-type H\textsuperscript{+}-ATPases are observed in the plasma membranes of EAT cells (30), osteoclasts (31), and macrophages (32). Overexpression of a plasma membrane H\textsuperscript{+}-ATPase in nonpnephel cells is, therefore, feasible. The consequences of such expression might lead to tumorigenesis, as evidenced by the current system. It must be remembered, however, that insertion of a H\textsuperscript{+}-ATPase into the plasma membrane will induce other effects besides increasing the magnitude of the pH gradient. Because this pump is electrogenic, these cells have a membrane potential of about ~40 mV, while the membrane potential of the parent cells is ~20 mV (16). Such a difference could cause significant changes in transport thermodynamics, and these changes could contribute to the transformed phenotype. Also, it is likely that these cells have an increased turnover rate of ATP, and this could lead to metabolic changes corresponding to transformation.

An example of this is glycolysis, which is probably a consequence of high ATPase rates. High rates of glycolysis are seen in both RNla and N-mut cells (Fig. 4f). Both of these have high ATPase rates; yet, only the H\textsuperscript{+}-ATPase of RNla cells pumps protons efficiently and, hence, gives a high pH\textsuperscript{x} (Fig. 2 A and B). Furthermore, the glycolytic rates are relatively insensitive to changes in the extracellular and hence, intracellular, pH (Fig. 4f). Glycolysis produces 1 ATP and 1 H\textsuperscript{+} per lactate. If (i) all ATP is the H\textsuperscript{+}-ATPase is provided by glycolysis, (ii) all glycolysis is excreted by the ATPase, and (iii) the stoichiometry of the ATPase is 1 H\textsuperscript{+} per ATP, then a futile cycle would result. The higher steady-state pH\textsuperscript{x} of the RNla cells rules out this possibility; yet, it does not preclude some "futile cycling" from occurring.

In summary, this system exhibits most traits of tumorigenic transformation: tumorigenicity in nude mice, soft agar growth, high-density growth, serum-independent growth, and high rates of aerobic glycolysis. This phenotype is the result of a single transfection with an active plasma membrane H\textsuperscript{+}-ATPase. Because the primary effects of this transfection are known, determining the events required for tumorigenic transformation is a tractable problem. This system raises the possibility that H\textsuperscript{+}-ATPases play a role in the etiology of carcinogenesis.

This work was supported by National Institutes of Health Grant ROI GM4046-01.

SECTION 2.

Metabolic effects of suramin on cultured mammalian cells

(Int. J. Cancer, Submitted).
METABOLIC EFFECTS OF SURAMIN ON CULTURED MAMMALIAN CELLS

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SUMMARY

Suramin is an effective anti-proliferative drug in vitro and in vivo, and is in clinical trials as a chemotherapeutic agent against prostate carcinoma. Its effects on growth are believed to be due to its inhibition of growth factor binding, yet this drug has a number of other activities. One of these is an inhibition of V-type H⁺ ATPase activity. This is important since expression of a plasma membrane H⁺-ATPase activity might be associated with tumorigenic transformation. We therefore tested the effects of suramin on pH⁰ regulation and other metabolic parameters in tumorigenic and non-tumorigenic murine cell lines grown in flasks or in suspension. To complement these studies, we also investigated the effects of N-ethylmaleimide (NEM) and fusidic acid, which have also been shown to
inhibit H⁺-ATPase activities. Our results indicate that, while these drugs may be effective inhibitors of the isolated V-type H⁺ ATPases, their effects on growth and pH in vivo can be explained by their effects on primary energy metabolism. Suramin stimulates O₂ consumption, inhibits glycolysis and induces cytosolic alkalinization. Suramin was more effective in inhibiting growth in the anchorage-dependent lines, with the tumorigenic cells being more sensitive. In contrast, fusidic acid and NEM inhibited cell growth equally in all cell lines. NEM and fusidic acid also inhibited O₂ consumption rates and caused acidification. These data clearly show that suramin has metabolic effects which are distinct from inhibition of growth factor binding and V-H⁺ ATPase inhibition, and suggest that caution should be exercised when interpreting mechanisms of action in vivo and in vitro.
Suramin, a polysulfonated polyaromatic, is a potent and impermeant anti-trypanocidal drug known to inhibit a variety of oxidative and hydrolytic enzymes (Wills and Wormall, 1950; Fortes et al., 1973). It suppresses growth of a number of cell lines in vitro (Spiegelman et al., 1987; Moscatelli et al., 1989; Jindal et al., 1990). There is renewed interest in this drug as a chemotherapeutic agent against HIV virus infectivity (Mitsuya et al., 1984) and against carcinoma of the prostate (Stein et al., 1986). The mechanism by which suramin exerts its anti-proliferative effects are unclear. It is commonly thought that its actions are mediated by its ability to bind to and inhibit the action of a variety of growth factors (Hosang, 1985; Coffey et al., 1987; Kopp and Pfeiffer, 1990). However, suramin inhibits a number of other biological activities, including Na\(^+\)+K\(^+\) ATPase (Fortes et al., 1973), glycolysis (Fantini et al., 1989), Ca\(^{2+}\)-ATPases (Calcaterra et al., 1988), G proteins (Butler et al., 1988), DNA polymerases (Jindal et al., 1990), and phosphoinositol and diacylglycerol kinases (Kopp et al., 1990). The terminal effects of this drug include not only inhibition of growth, but also inhibition of cell invasion (Nakajima et al., 1991) and modulation of differentiation (Fantini et al., 1989). This drug has also been reported to inhibit the activity of vacuolar proton-translocating ATPases (V-H\(^+\) ATPase) (Moriyama and Nelson, 1988).
Although normally confined to endosomes and lysosomes, V-H\(^+\) ATPases can also be expressed on the plasma membranes of some mammalian cells, such as macrophages (Swallow et al., 1990; Swallow et al., 1988), osteoclasts (Blair et al., 1989), and tumor cells (Stone et al., 1989; Heinz et al., 1981) including primary human tumor cells (Martínez-Zaguilán et al., 1992). Expression of plasmalemmal H\(^+\) ATPase activity could be important to tumorigenicity, since transfection of 3T3 cells with yeast plasma membrane H\(^+\) ATPase (P-H\(^+\) ATPase) induces these phenotypically normal cells to be tumorigenic (Perona and Serrano, 1988). This is thought to occur by chronically raising the intracellular pH (pH\(^in\)) (Gillies et al., 1990). Although a high pH\(^in\) is thought to only be permissive for cell proliferation (Gillies et al., 1990; Gillies and Martínez-Zaguilán, 1991; Moolenaar, 1986; Grinstein et al., 1989; Bierman et al., 1988; Lucas et al., 1988; Szwergold et al., 1989), these observations argue that chronic supraphysiological pH\(^in\) can induce a tumorigenic phenotype. We therefore hypothesized that suramin might be exerting its antiproliferative effects as a H\(^+\) ATPase inhibitor in those cell lines expressing plasmalemmal H\(^+\) ATPase activity.

In this study, we have tested the effects of suramin on pH\(^in\) regulation, cell growth, and energy metabolism in non-tumorigenic (3T3 and CHO) and tumorigenic (EAT and RN1a cells) mammalian cells. The rationale for selecting these cell lines is based on the fact that 3T3 cells and CHO cells are non tumorigenic and exhibit
strong degree of density dependent inhibition. In contrast, EAT cells and RN1a cells are highly tumorigenic and contain, respectively, V-type (Stone et al., 1989; Heinz et al., 1981) and P-type (Perona and Serrano, 1988; Gillies et al., 1990) H\(^+\) ATPase activities in their plasma membranes. The 3T3 and RN1a are anchorage dependent for growth, whereas CHO and EAT are grown in suspension. The effects of suramin are compared to those of other H\(^+\) ATPase inhibitors, N-ethyl maleimide (NEM) and fusidic acid.

Results indicate that suramin's effects are not mediated by inhibition of H\(^+\) ATPase or growth factor binding. Instead, it appears that the primary effect of this drug is on cellular energy metabolism. In contrast to NEM and fusidic acid, suramin stimulates O\(_2\) consumption in a dose-dependent fashion. No differences in \textit{in situ} ATP content were observed between suramin treated and control cells. Concomitant with the increase in O\(_2\) consumption, suramin inhibits lactate production and hence reduces the amount of energy production from glycolysis.

These results indicate that suramin either increases or does not change energy demand in cells. This is distinct from its reported activities, which indicate that the drug is a generalized ATPase inhibitor. The increased energy demand is met by an increase in respiration, which is significant in light of tumor cell metabolism.
MATERIAL AND METHODS.

**Fluorescence Measurements.** All fluorescence measurements were performed in a temperature-controlled unit housed in an SLM8000C (SLM. Urbana, ILL), at 37°C using 4 nm slits and an external rhodamine standard as a reference.

**Culture of Cell Lines.** Non tumorigenic BALB/c-3T3 embryo fibroblasts were obtained from American Type Cell Culture Collection, Rockville, MD (ATCC CCL 163). The pH\textsuperscript{th} regulatory mechanisms of BALB/c and NIH-3T3 cells are essentially identical. NIH 3T3 cells, transfected with yeast H\textsuperscript{+}-ATPase (i.e. RN1a cells, tumorigenic) were provided by Dr. Rosario Perona (Instituto de Investigaciones Biomedicas, Madrid, Spain). These cells were cultured as fully described in Gillies et al (1990). For pH\textsuperscript{th} measurements, cells were subcultured on glass coverslips (9 x 22 mm), in 100 mm-dish at 5 x 10\textsuperscript{4} cells/dish.

CHO cells were generously provided by Dr. Bruce Dale (Texas A&M Univ.). These cells were subcultured in SMEM (Gibco, Grand Island, NY) as suspension cultures, supplemented with 10% Nu-serum, in a 5% CO\textsubscript{2} atmosphere at 37°C. Stocks were grown in a 250 ml spinner flask, and passed bi-weekly at an inoculation density of 8 x 10\textsuperscript{9}/ml. For experiments, cells were seeded at 2 x 10\textsuperscript{5}
in a 1 L spinner flask.

EAT cells were obtained from American Type Culture Collection (ATCC CCL 77). These cells were cultured in SMEM media, as suspension cultures, essentially as described for CHO cells, except that they were supplemented with 10% FBS (Hyclone) instead of 10% Nu-Serum. Control studies have shown that Nu-Serum is unable to support suspension growth in this cell line. For experiments they were handled as described for CHO cells.

Buffers. Cell Suspension Buffer (CSB) contained: 1.3 mM CaCl₂, 1 mM MgSO₄, 5.4 mM KCl, 0.44 mM KH₂PO₄, 110 mM NaCl, 0.35 mM NaH₂PO₄, 5 mM glucose, 2 mM glutamine and 25 mM HEPES, at a pH of 7.15 at 37°C. High K⁺ buffer (High K⁺) contained: 5 mM glucose, 2 mM glutamine, 10 mM HEPES, 10 mM MES, 10 mM Bicine and the corresponding intracellular K⁺ concentration for each cell line (see table 1 for results on intracellular K⁺ and cell volume measurements).

Culture Preparation for pH⁻⁻ measurements. Subconfluent 3T3 and RN1a cell cultures grown onto coverslips were loaded with 2 μM BCECF, as described fully in Gillies, et al (1990). Sample temperature was maintained at 37°C by keeping both the water jacket and the perfusion buffer at 37°C using a circulator water
bath (Lauda Mod. RM20, Brinkmann Instruments Co.).

For fluorescence measurements in suspension cells (CHO and EAT cells), aliquots of cells growing in spinner flasks were removed as needed, and were centrifuged at 350 xg for 5 min at 4°C. The pellet was resuspended in the appropriate buffer. Aliquots of 2 \times 10^6 cells/3.0 ml (fluorometer cuvette) were used for pH$_\text{in}$ measurements. Cell loading with BCECF was performed essentially as described for attached 3T3 cells (Gillies et al., 1990), except that after the second incubation, cells were washed again to remove excess dye. Fluorescence measurements were performed in a temperature-controlled continuously stirred cuvette.

**pH$_\text{in}$ Measurements using BCECF.** Data were collected in a continuous acquisition mode, where the emission was set at 529 nm with excitation wavelengths of 435, 450 and 500 nm. The fluorescence at 435 nm represents the isoexcitation point for this dye. This cycle lasts 3.68 seconds, and was repeated as many times as necessary. The ratio of fluorescence intensities at excitations at 450 and 500 nm was converted to pH$_\text{in}$ values using eqn. [1].

\[
\text{pH} = \text{pK}_a + \log \left( \frac{S_{b2}}{S_{b2}} \right) + \log \left[ \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right] \quad \text{eqn. [1]} \quad (\text{Martínez-Zagullán et al., 1991})
\]
where $R_{obs}$ is the observed ratio at any given pH; $R_{min}$ is the ratio obtained when the dye is fully protonated, and $R_{max}$ represents the ratio of fluorescence obtained when the dye is fully unprotonated. $S_{fb}$ and $S_{bz}$ are the fluorescence values for the free and bound forms of the dye, respectively, at the denominator wavelength. The quantities $S_{fb}/S_{bz}$ are used to correct for the fact that the denominator wavelength is ion-sensitive.

Data were translated to ASCII format. Conversions of ratio values to $pH_{in}$ was performed with Lotus 1-2-3 (Lotus Development Corp.) by using equation [1], and data were plotted by using a commercial computer software (Sigmaplot, Scientific Graph System by Jandel, Sci.).

In Situ Calibration of BCECF. Calibration of intracellular BCECF into the cells was performed as described previously (Gillies et al., 1990). From these in situ calibration curves we obtained the $R_{max}$, $R_{min}$ and $pK_a$ for this dye in each cell type (cf Martínez-Zaguilán et al., 1991) (Table 1).

Cell Number. Cell number was determined using a dye binding assay, as described in Gillies et al (1986). For these experiments we took advantage of the fact that, in high Ca$^{2+}$ media, both CHO and EAT cells attach to tissue culture plastic. This was achieved by substituting SMEM which is a low Ca$^{2+}$ media, by
MEM which contains 1.8 mM Ca\(^{2+}\). Briefly, all cells used in this study were plated at 3 \(\times\) 10^4 in 24-well plates (Flow Labs). Twelve hours later suramin, fusidic acid, and NEM were added to the media at various concentrations (10 nM - 2 mM). At 24, 48 and 72 hours thereafter, the medium was removed, and the cells were washed with CSB at 37°, fixed with 1% glutaraldehyde, and then stained with 0.1% crystal violet. Subsequently, the dye was solubilized in 0.2% Triton X-100. The absorbance at 590 nm of this Triton X-100 solution is linearly related to cell number.

**Oxygen Consumption Measurements.** Oxygen consumption measurements were performed using a Clarke-style electrode (YSI Model 5300 Biological Oxygen Monitor, Yellow Spring Instruments, CO. Inc.). Briefly, aliquots of EAT and CHO cells grown as suspension cultures were centrifuged at 350 xg for 5 min and the pellet was resuspended at 2x10^7 / 6 ml of CSB, pH\(^{\text{ex}}\) = 7.15. This cellular suspension was then placed in a thermostatted, continuous stirred cell chamber, at 37°C to measure O\(_2\) consumption in the presence and absence of suramin, NEM and fusidic acid at various concentrations. Recordings were obtained and the slopes \((dO_2/dt)\) before and after treatment were calculated by linear regression analysis by using a commercially available software (MINSQ, MicroMath Scientific Software, Salt Lake, UT.). The effect of the drug-treatment on O\(_2\) consumption can be estimated by:
%I or %S = \left\{ \frac{(dO_2/dt)_{a1} \cdot (100)}{(dO_2/dt)_{b1}} \right\} - 100 \quad \text{eqn [2]}

where %I or %S represents the percentage of inhibition or stimulation, respectively; \( (dO_2/dt)_{b1} \) represents the slope determined within the first 5 minutes before treatment (internal control); and \( (dO_2/dt)_{a1} \) is the slope after treatment (within the next 10 minutes).

Chemicals and Drugs. Suramin was obtained from Mobay Chem. Corp. (FBA pharmaceuticals, NY), and was prepared as a sterile stock solution of 0.1M in deionized H\(_2\)O, protected from the light and stored at -20°C, until used. Fusidic acid, NEM and DMSO were obtained from Sigma Chemical (St. Louis, MO). Fusidic acid was prepared as a sterile solution of 4M in deionized H\(_2\)O. NEM was dissolved in DMSO and protected from the light. Working solutions of Fusidic acid and NEM were always prepared from stock immediately before the experiments. BCECF was obtained from Molecular Probes (Eugene, OR). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical.

Statistical Analyses. Statistical significance of our data were calculated using a Student t-test for unpaired or paired data. The IC\(_{50}\)'s for the effect of fusidic acid, NEM, and suramin on pH\(_{10}\), O\(_2\) consumption and cell growth were obtained directly from the titration curves, by non linear least squares regression analysis.

**NMR measurements.** NMR spectra were acquired on a Bruker AMX 400 WB spectrometer, containing a 25 mm broadbanded probe, custom made to accommodate bioreactors (Spectrospin, Inc) Unless otherwise noted, spectra were obtained by applying a 30° pulse every 1.0 sec with 5 watts composite (WALTZ-16) $^1$H decoupling during acquisition. Spectra were accumulated for 1 hour for 10 days prior to and six days following suramin treatment.

The bioreactor circuits used in these experiments were similar to those described previously (Gillies *et al.*, 1991). The temperature in the bioreactor was maintained at 36.5° ± 0.5°C throughout the experiment. The bioreactor itself was of the same essential design as before, except that it was modified to contain all ports for inlet, outlet and inoculation, at one end of the reactor. As before, the reactors were constructed by Microgon, Inc. (Laguna Hills, CA).

Prior to inoculation into the bioreactor, the RN1a cells were inoculated into gelatin beads (HyClone, Inc) and maintained in suspension for at least two days prior to inoculation into the bioreactor. These and all subsequent media contained 2.5 mM dimethylmethylphosphonic acid (DMMP) as a chemical shift reference at 36.8
Cells were inoculated into the bioreactor by siphoning 2 L of the cell or cell-containing bead suspensions into the reactor over a period of 1-2 hrs. Under these conditions, the initial inoculum contains a total of ca. $10^9$ cells in a total volume of 16 ml. High-quality $^{31}$P spectra could be accumulated in 2 hours under these initial conditions. As the cultures grew, the time resolution increased such that, at maximum density, similar spectra could be accumulated in 1-2 mins. The initial bleed-and feed (B/F) rates in the circuit were 500 ml day$^{-1}$. Thereafter, the B/F rate was iteratively increased so as to maintain the medium pH above 7.2. Initial aeration conditions were 5% CO$_2$ / 95% air. Once global ATP levels reached 0.5 mM relative to DMMP at 2.5 mM, the aeration was changed to 45% O$_2$ : 5% CO$_2$ : 50% N$_2$. When global ATP levels reached 1.0-1.5 mM, the aeration mix was changed to 60% O$_2$ : 5% CO$_2$ : 35% N$_2$. This steady-state was maintained for two days prior to the addition of suramin. Samples of the media were taken twice a day and analyzed for glucose and lactate concentrations using an automated glucose/lactate analyzer (Yellow Springs Instruments model 2000).
RESULTS.

Suramin inhibits vacuolar-type H\(^+\)-ATPases \textit{in vitro} through inhibition of ATP catalysis (Moriyama and Nelson, 1989). We have tested for the effect of this drug on pH\textsubscript{in} regulation in tumorigenic (EAT and RN1a) and non tumorigenic (3T3 and CHO) mammalian cells. Our results shown that only RN1a cells respond with acidification upon treatment with this drug at concentrations lower than 0.5 mM (Fig 1). The maximum cytosolic acidification was observed at 100 \(\mu\text{M}\), with an estimated IC\(_{50}\) of 50 \(\pm\) 12 \(\mu\text{M}\). At concentrations > 0.5 mM an alkalinization was observed in RN1a cells (Fig 2A). In contrast, suramin treatment in EAT, CHO and 3T3 cells, resulted in cytosolic alkalinization at all concentrations tested (cf Fig 1 and 2). The calculated EC\(_{50}\)'s for the suramin-induced alkalinization were as follows: EAT, 75 \(\pm\) 18 \(\mu\text{M}\); CHO, 350 \(\pm\) 53 \(\mu\text{M}\); and 3T3, 200 \(\pm\) 68 \(\mu\text{M}\). In contrast, the EC\(_{50}\) for alkalinization in RN1a cells was considerably higher: 600 \(\pm\) 85 \(\mu\text{M}\). Suramin concentrations < 100 \(\mu\text{M}\) did not affect pH\textsubscript{in} in 3T3, CHO or EAT cells (Fig 2A,B and data not shown).
FIGURE 1. Effect of suramin on pH\textsuperscript{in}. Effect of 0.25 mM suramin on pH\textsuperscript{in} of 3T3, RN1a, CHO and EAT cells. Cells were loaded with BCECF as described in Methods, and the fluorescence ratio produced at excitation wavelengths of 500 and 450 nm (hv = 529) was continuously monitored. 3T3 and RN1a cells were continuously perfused with CSB, pH\textsuperscript{ex} = 7.15. CHO and EAT cells were used as suspension cells and were continuously stirred in the fluorometer cuvette. In all cases the buffer lacked HCO\textsubscript{3}\textsuperscript{-} and serum. The arrow indicates the change of the perfusate and/or direct addition of the drug into the fluorometer cuvette from a concentrated stock.
FIGURE 2. Titration of the effect of suramin on pH in in attached cells (A) and suspended cells (B). Handling of these cells was performed as described in legend to Fig 1. ΔpH in was evaluated by determining the steady state pH in before treatment and subtracting the pH in after treatment (cf Fig 1). Data represent the mean ± S.D. of 3-5 experiments per data point, and were obtained from a series of experiments similar to those shown in figures 1 (see Materials and Methods). Statistical analysis was performed by using Student's t-test. ***P < 0.0005 3T3 versus RN1a and CHO versus EAT; otherwise differences are not significant.
We also evaluated the effect of fusidic acid and NEM on pH\(_{\text{in}}\) regulation in tumorigenic and non tumorigenic mammalian cells. These drugs also inhibit V-H\(^+\) ATPase activity, albeit by different mechanisms (Moriyama and Nelson, 1988; Stone \textit{et al.}, 1989; Moriyama and Nelson, 1989; Bowman and Bowman, 1986; Davenport and Slayman, 1988). Fusidic acid inhibits H\(^+\) transport and uncouples V-H\(^+\)-ATPase. At 0.25 mM this drug decreases pH\(_{\text{in}}\) in EAT, CHO and RN1a cells (figure 3). In contrast, 0.25 mM fusidic acid has only a minor effect on the pH\(_{\text{in}}\) of 3T3 cells. In CHO, EAT and RN1a cells, fusidic acid induced a cytosolic acidification at all concentrations between 50 \(\mu\text{M}\) to 2 mM, while the effects on 3T3 cells were insignificant (Fig 4). Concentrations greater than 2 mM did not produce larger acidifications. The decrease in pH\(_{\text{in}}\) was much greater for CHO and EAT cells. CHO and EAT cells have a resting pH\(_{\text{in}}\) 0.2 pH units higher than that found in RN1a cells (Table 1). The data from Figs 4A and 4B were used to estimate the IC\(_{50}\)'s of 175 ± 30 \(\mu\text{M}\) for RN1a cells; 204 ± 28 \(\mu\text{M}\) for CHO cells; and 408 ± 37 \(\mu\text{M}\) for EAT cells.
FIGURE 3. Effect of fusidic acid on pH<sup>m</sup>. Effect of 0.25 mM fusidic acid on pH<sup>m</sup> of 3T3, RN1a, CHO and EAT cells. Cells were handled essentially as described in Fig 1.
FIGURE 4. Titration of the effect of fusidic acid on pH\textsuperscript{in} in attached cells (A); and suspended cells (B). Handling of these cells was performed as described in legend to Fig 1. Δ pH\textsuperscript{in} was evaluated as described in legend to Fig 2 (see Materials and Methods). Statistical analysis was performed by using Student's t-test.

*P < 0.05 3T3 versus RN1a; **P < 0.0005 3T3 versus RN1a and CHO versus EAT. Unreported differences are not significant.
Figure 5 shows that 0.25 mM NEM induced an acidification in EAT, CHO and RN1a cells, but not in 3T3 cells. Figures 6A and 6B summarize the results for the titration of the effect of NEM on pH\text{intracellular} in 3T3, RN1a, CHO, and EAT cells. NEM induces cytosolic acidification in RN1a, CHO and EAT cells. In 3T3 cells, a slight acidification was observed only at 0.25 mM while higher concentrations of this drug tended to alkalinize. Similar pH\text{intracellular} increases were observed in EAT, CHO and RN1a cells at concentrations greater than 1 mM (data not shown), suggesting that NEM has biphasic effects, i.e. it decreases pH\text{intracellular} at lower concentrations, while at concentrations \( \geq 1 \) mM it increases pH\text{intracellular}. The estimated IC\text{50} values for the effect of NEM-induced cytosolic acidification in these cells, as determined from the data in Fig 6 were as follows: EAT cells, IC\text{50} = 159 \pm 36 \mu M; CHO cells, IC\text{50} = 220 \pm 85 \mu M; RN1a cells, IC\text{50} = 290 \pm 51 \mu M.
FIGURE 5. Effect of NEM on pH\textsuperscript{in}. Effect of 0.25 mM NEM on pH\textsuperscript{in} of 3T3, RN1α, CHO and EAT cells. Cells were handled essentially as described in Fig 1.
FIGURE 6. Titration of the effect of NEM on pH\textsuperscript{inh} in attached cells (A); and suspended cells (B). Handling of these cells was performed as described in legend to Fig 1. D pH\textsuperscript{inh} was evaluated as described in legend to Fig 2 (see Materials and Methods). Statistical analysis was done by using Student's t-test. *P < 0.05 CHO versus EAT; **P < 0.005 CHO versus EAT; ***P < 0.0005 3T3 versus RN1a and CHO versus EAT.
It is possible that, at high concentrations, these drugs could have an effect on mitochondrial $F_1/F_0$ ATPase and that this could lead to a perturbation of $pH_{int}$. We therefore measured the effects of these drugs on $O_2$ consumption in the suspension cells used in this study (i.e. CHO and EAT cells). As shown in Fig 7A, suramin induced a significant increase in the rate of oxygen consumption in both EAT and CHO cells with an apparent $EC_{50}$ of $226 \pm 61 \, \mu M$ and $304 \pm 84 \, \mu M$, respectively. In contrast, both NEM and fusidic acid caused a significant decrease in the rate of oxygen consumption (Fig 7B, and 7C). The $IC_{50}$'s calculated from these experiments were $41 \pm 11 \, \mu M$, and $39 \pm 12 \, \mu M$ for the effect of NEM on oxygen consumption in EAT and CHO cells, respectively (Fig 7B). Since NEM is a general sulfhydryl reagent, it could be affecting a number of other mitochondrial proteins (Swallow et al., 1988; Swallow et al., 1990; Davenport and Slayman, 1988; Grinstein et al., 1985). For the effect of fusidic acid on oxygen consumption (Fig 7C), $IC_{50}$ values of $136 \pm 32 \, \mu M$ and $135 \pm 35 \, \mu M$ were determined in EAT and CHO cells, respectively. The effects of fusidic acid are probably mediated through the H$^+$ ATPase (Moriyama and Nelson, 1988).

The effects of suramin on respiration are curious. A possible explanation for the effects of this drug on $O_2$ consumption is that it is altering the energetics of the cell by either increasing ATP demand (with no concomitant increase in glycolysis) or that it is inhibiting glycolysis. Figure 8 illustrates in vivo $^{31}$P NMR spectra of
RN1a cells before and after five days treatment with suramin. Although profound changes in the amount of mobile phospholipids are observed, suramin has no effect on the steady-state levels of ATP, even after chronic treatment. This suggests that, if suramin does affect cell energetics, its effects do not involve a change in ATP concentrations. Therefore, since oxidative ATP production is increased, either the ATP demand must be concomitantly increased or non-oxidative ATP production (e.g. glycolysis) must be concomitantly inhibited. As shown in figure 9, glycolytic ATP production in RN1a cells is significantly inhibited by this drug. These data are consistent with previous reports by Fantini et al (1989).
FIGURE 7. Effect of suramin, fusidic acid and NEM on oxygen consumption. 2 x 10^7 cells/6 ml CSB (final volume) were placed in an oxygen chamber and oxygen consumption was measured using a Clark style electrode (see Materials and Methods). After the initial 5 minutes of recording (i.e. steady state O_2 consumption), drugs at the final concentrations indicated were added into the chamber using a Hamilton syringe and the O_2 consumption was recorded for an additional 10 minutes. Thereafter, the difference between the steady-state O_2 consumption and the post-steady state O_2 consumption (i.e. after drug treatment), was determined by using eqn [2] (see Materials and Methods). Each point represent the mean ± S.D. of 7 determinations.
FIGURE 8. 31-P NMR spectra of RN1a cells treated with suramin. RN1a cells were grown in gelatin beads and inoculated into hollow fiber bioreactors, as described in Methods. $^{31}$P spectra were accumulated periodically for 10 days to monitor growth and pH regulation. After this time, the perfusate was changed for one containing suramin at a concentration of 100 μM. These conditions were maintained for five days, after which the medium was changed back to suramin-free. These spectra were obtained just prior to (lower) and five days after (upper) addition of suramin. Abbreviations: PME = phosphomonomesters, including phosphorylethanolamine and phosphorylcholine; P_i = inorganic phosphate; PL = mobile phospholipids; PCR = phosphocreatine; ATP-$\alpha$, -$\beta$ and -$\gamma$ = terminal, primary and middle phosphates of ATP; UDPH = Uridine diphosphoryl glucose/galactose; NADP = nicotinamide adenine dinucleotide (phosphate).
All three of these drugs inhibit growth in these cell lines. Figure 10A shows the effect of suramin (dotted regression line) and fusidic acid (solid regression line) on cell growth in anchorage-dependent cells which have a H⁺-ATPase, i.e. RN1a (solid symbols), and in their non-tumorigenic counterparts, 3T3 cells (open symbols). These data were obtained after 72 hours of growth in the continuous presence of the indicated drug. These data show that RN1a cells are more susceptible to growth inhibition by suramin than are 3T3 cells, and that neither CHO nor EAT cells were susceptible to cell growth inhibition by this drug. Furthermore, we have evaluated each drug at various time points including 48, and 72 hours in order to obtain the IC\textsubscript{50}'s for each one of the cell lines studied. The results of such analyses are expressed in table 2. Since no complete suppression of cell growth by suramin was observed in either CHO or EAT cells, the IC\textsubscript{50}'s for such effects were not obtained (c.f Fig 10B). In fact, even at concentrations as high as 5 mM, less than 50% inhibition was consistently observed (data not shown).

The NMR data suggest that suramin is not cytotoxic, since no decline in viable cell number is observed. To follow up, we monitored the reversibility of suramin effects on cell number in 24-well cultures. As shown in figure 11, the effects of suramin on cell growth are reversible, indicating that it is cytostatic, rather than cytotoxic. This is consistent with its effects on cell metabolism and differentiation.
FIGURE 9. Effect of suramin on glucose consumption and lactate production. Media from NMR experiments were collected at time points prior to and following addition of suramin, and analyzed for glucose and lactate levels as described in Methods. Glucose levels in perfusate were subtracted from glucose levels in medium to determine glucose consumption. Data are presented as mM glucose consumed or lactate produced. The lower curve represents the lactate/glucose ratio. The open bar indicates the length of time that suramin was present in the perfusate at a concentration of 100 μM.
FIGURE 10. Cell Growth. Cells were grown in 24-miniwell plates and cell number determined by crystal violet as previously described (Gillies et al., 1986). 24 hours after inoculation, media was exchanged for one containing different concentrations of the drug as indicated. Cells were continuously exposed to the drug for a period of 72 hours. Therefore, the cell growth achieved after 72 hours in culture, in the absence of the drug (control) is considered as 100% cell growth. Addition of drug results in a concentration dependent decrease in cell growth, when compared to the control at 72 hrs. Data represent the mean ± S.D. of 3 experiments. Dotted regression lines = suramin; solid regression lines = fusidic acid.
FIGURE 11. Reversible inhibition of cell growth by suramin. Cells were plated in 24-well plates, and cell number was determined at time points by dye binding, as described elsewhere (Gillies et al., 1986). All cells were grown for 24 hours in the absence of suramin. After one day of growth (time 0), three sets were treated with suramin at 100 μM. Two of these sets were incubated with suramin for 24 and 48 hours (closed circles), after which cells were refed with suramin-free medium (open circles). The third set was continuously grown in the presence of suramin for 72 hrs (closed circles). Data represents the mean ± S.D. of 8 determinations.
TABLE 1. *IN SITU* CALIBRATION PARAMETERS AND $pH^{in}$

<table>
<thead>
<tr>
<th></th>
<th>BALB/c</th>
<th>RN1a</th>
<th>CHO</th>
<th>EAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_a$</td>
<td>6.95 ± 0.02</td>
<td>6.96 ± 0.06</td>
<td>6.95 ± 0.02</td>
<td>7.06 ± 0.04</td>
</tr>
<tr>
<td>$R_{min}$</td>
<td>1.08 ± 0.04</td>
<td>0.99 ± 0.08</td>
<td>1.19 ± 0.04</td>
<td>1.12 ± 0.05</td>
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<tr>
<td>$R_{max}$</td>
<td>6.88 ± 0.07</td>
<td>6.50 ± 0.19</td>
<td>6.19 ± 0.03</td>
<td>5.90 ± 0.12</td>
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<td>$pH^{ia}$</td>
<td>6.75 ± 0.12(42)</td>
<td>7.26 ± 0.12(47)</td>
<td>7.44 ± 0.07(39)</td>
<td>7.41 ± 0.11(41)</td>
</tr>
<tr>
<td>$Vol^{in}$</td>
<td>9.34 ± 0.3</td>
<td>6.45 ± 0.19</td>
<td>6.45 ± 0.19</td>
<td>5.78 ± 0.91</td>
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<tr>
<td>$K^{+(in)}$</td>
<td>146 ± 28</td>
<td>140 ± 25</td>
<td>144 ± 20</td>
<td>163 ± 52</td>
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</tbody>
</table>

Anchorage-dependent (BALB/c and RN1a) and suspension (CHO and EAT) cells were grown and handled for fluorescence measurements as described in Material and Methods. The *in situ* calibration parameters were obtained by using equation [1] (c.f. Material and Methods), and represent the mean ± S.D. of 35-48 independent pH measurements within the pH range from 6 to 8. These parameters were used to calculate the steady-state $pH^{in}$. The resting $pH^{in}$ was obtained in CSB, HCO$_3$-free media, at $pH^{is}=7.15$, and represents the mean ± S.D. of the number of experiments indicated in parenthesis. Intracellular cell volume ($Vol^{in}$; nL/μg protein), and intracellular $K^+$ measurements ($K^{+(in)}$; mM), were performed as described by Giuliano and Gillies (1987).
TABLE 2. IC$_{50}$'s OF H$^+$-ATPase INHIBITORS ON CELL GROWTH (µM)

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>SURAMIN</th>
<th>FUSIDIC ACID</th>
<th>NEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>325.2 ± 35.3</td>
<td>229.3 ± 94.1</td>
<td>0.78 ± 0.25$^{***(c)}$</td>
</tr>
<tr>
<td>RN1a</td>
<td>108.2 ± 12.8$^{***(a)}$</td>
<td>62.3 ± 9.4$^{***(b)}$</td>
<td>2.50 ± 0.58</td>
</tr>
<tr>
<td>CHO</td>
<td>N.A.</td>
<td>238.1 ± 77.2</td>
<td>2.01 ± 0.72</td>
</tr>
<tr>
<td>EAT</td>
<td>N.A.</td>
<td>100.8 ± 15.5$^{**(b)}$</td>
<td>2.05 ± 0.61</td>
</tr>
</tbody>
</table>

Cells were grown in 24-miniwell plates and cell number determined by crystal violet as previously described (Gillies et al., 1986). Time points at 48 and 72 hrs were plotted as shown in Fig. 27 and the IC$_{50}$ was determined from such curves (see Materials and Methods). Data represent the mean ± S.D. of 6 experiments. N.A. = not applicable, since suramin did not suppress >50% cell growth in these cell lines at any of the concentrations tested ranging from 0 - 5 mM. Statistical analysis was performed by using Student's t-test (Snedecor and Cochran, 1972). $^{***(a)}$P < 0.0005 RN1a versus BALB/c; $^{***(b)}$P < 0.0005 RN1a versus BALB/c; $^{**(b)}$P < 0.005 EAT versus CHO; $^{**(b)}$P < 0.0005 BALB/c versus RN1a, CHO, and EAT.
DISCUSSION.

There is evidence suggesting the presence of V-H$^+$ ATPases in the plasma membrane of several cell types including EAT (Stone et al., 1989; Heinz et al., 1981), hepatocytes (Ehrhardt, 1984), macrophages (Swallow et al., 1988; Swallow et al., 1990), osteoclasts (Blair et al., 1989), and epithelial cells (Turrini et al., 1989). Acid secreting cells of the urinary tract contain P-type H$^+/K^+$ ATPases, which are not constitutively present in the plasma membrane, yet are translocated from an intracellular reservoir through an exocytotic process triggered by Ca$^{2+}$ (van Adelsberg and Al-Awqati, 1986). In EAT cells, a V-H$^+$ ATPase seems to be constitutively present in the plasma membrane and may play a significant role in maintaining a chronically alkaline pHe$^{in}$ (Stone et al., 1989). Furthermore, transfection of normal 3T3 cells with a yeast P-type H$^+$-ATPase renders these cells tumorigenic (Perona and Serrano, 1988), suggesting that some mammalian cells might be tumorigenic by virtue of the constitutive presence of a H$^+$-ATPase that contributes to the maintenance of a high pHe$^{in}$ (Gillies et al., 1990). In order to test this hypothesis, specific inhibitors for H$^+$-ATPases are required. Although the inhibitors for these enzymes (suramin, fusidic acid, and NEM) are relatively non-specific (Moriyama and Nelson, 1988; Moriyama and Nelson, 1989; Swallow et al., 1988; Swallow et al., 1990; Stone et al., 1989; Bowman and Bowman, 1986; Davenport and Slayman, 1988; Turrini et al., 1989; Grinstein et al., 1985), we have
used them to ask whether we could discriminate tumorigenic from non-tumorigenic cells on the basis of their sensitivities to these drugs. Our results indicate that the effects of both fusidic acid and NEM are consistent with their effect on cellular respiration. Inhibition of oxidative phosphorylation will invariably cause cellular de-energization and/or an increase in glycolytic rate. Either of these sequelae would lead to cytosolic acidification (Gillies et al., 1982).

The effects of suramin are less clear. Suramin affects pH\textsubscript{in} in all cell lines tested. However, only the RN1a cells responded with cytosolic acidification, whereas an increase in pH\textsubscript{in} was consistently observed in all the other cell lines (BALBc, CHO and EAT) (Figs. 1, 2A and 2B). A cytoplasmic alkalinization was also observed in RN1a cells treated with higher concentrations of suramin (Fig 2B). These results are curious since RN1a cells contain a P-type H\textsuperscript{+} ATPase, and suramin is reported to inhibit V-type H\textsuperscript{+}-ATPases (Moriyama and Nelson, 1988; Moriyama and Nelson, 1989). This increase in pH\textsubscript{in} may be the result of the inhibition of glycolysis, which would lead to decreased metabolic H\textsuperscript{+} production. In support of this, we have observed that decreased medium glucose levels causes higher intracellular pH values in these cells (data not shown). It is interesting to note that, although suramin increases glucose consumption rates, concomitantly inhibits the production of lactate (figure 9). The increase in O\textsubscript{2} consumption is not sufficient to account for the increased amount of glucose consumed. The fate of
the extra glucose is unknown, but may involve synthesis of phospholipids, as shown in the NMR spectra (Fig 8). This is similar to the activities of drugs which alter cytosolic redox. Therefore, our data indicate that suramin does not act as a V-type H\(^+\)-ATPase inhibitor \textit{in vivo}, and further suggest that this drug has other, undefined metabolic effects.

Our data shows that suramin addition to the culture media inhibited growth of RN1a more than 3T3 cells (cf Fig 10A). This is of further relevance since this drug also preferentially decreased pH\text{\textnormal{\textsuperscript{inh}}} in RN1a, but triggered an alkalinization in 3T3 cells (Fig. 1, and 2A). Similar results on the effect of suramin on suppression of cell growth have been reported elsewhere (Spiegelman et al., 1987; Moscatelli et al., 1989; Jindal et al., 1990; Hosang et al., 1985; Coffey et al., 1987; Fantini et al., 1989). In contrast, suramin was cytostatic, as opposed to cytotoxic, in CHO and EAT cells (Figs 10B and 11). Nakajima et al (1991) observed that suramin inhibited invasiveness, but not growth, in a melanoma cell line.

As shown in Fig. 10A and B, fusidic acid affects cell growth in both tumorigenic and non-tumorigenic cell lines. Importantly, the IC\textsubscript{50}’s obtained were higher for non-tumorigenic cell lines (Table 2). However, the concentrations required to inhibit cell growth are higher than those needed to decrease either pH\text{\textnormal{\textsuperscript{inh}}} or O\textsubscript{2} consumption. It is therefore unclear whether inhibition of cell growth is mediated
through inhibition of H$^+$-ATPase or through their effects on metabolism.

In contrast, NEM did not differentially affect cell growth in tumorigenic and non-tumorigenic cell lines, since similar IC$_{50}$'s were obtained for all cell lines (see Table 2). It is noteworthy to mention that the concentrations required to totally abolish cell growth in most cell lines was less than 5 $\mu$M. Therefore, the effect of NEM on cell growth can not be accounted by either the decrease in oxygen consumption (Fig. 7B), or its effects on pH$_{in}$ since the concentrations required to affect these parameters are one to two orders of magnitude higher than those required to suppress cell growth (cf Fig. 5, 6, 10 and table 2).

In the present study we have identified some clear differences between the sensitivities of tumorigenic and non-tumorigenic cells to suramin, fusidic acid and NEM regarding their effects on pH$_{in}$, cell growth and oxygen consumption. It is apparent that these drugs lack the specificity required to employ them as H$^+$ ATPase inhibitors. Suramin decreases pH$_{in}$ only in RN1a cells whereas it increases pH$_{in}$ and O$_2$ consumption in all the other cell types. In contrast, fusidic acid and NEM decrease pH$_{in}$ and O$_2$ consumption in all cell lines. The correlation between O$_2$, pH$_{in}$ changes and cell growth is not clear. Furthermore, the biphasic pH$_{in}$ responses of 3T3 cells to NEM and RN1a cells to suramin clearly indicate that these drugs are affecting different pathways. Due to the renewed clinical
interest in suramin as a potential anticarcinogenic drug, we feel that caution should be exerted when interpreting the data from these clinical trials, since the mechanism of action of this drug is far from being understood.
REFERENCES


SECTION 3.

Involvement of a plasma membrane localized vacuolar type H⁺-ATPase in the maintenance of a high intracellular pH in human tumor cells.

INVOLVEMENT OF A PLASMA MEMBRANE LOCALIZED VACUOLAR TYPE H⁺-ATPASE IN THE MAINTENANCE OF A HIGH INTRACELLULAR pH IN HUMAN TUMOR CELLS.

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SUMMARY

Insertion of a yeast H⁺-ATPase in the plasma membrane of normal NIH-3T3 cells chronically raises their intracellular pH (pH\text{in}) and renders them tumorigenic (Perona and Serrano, Nature, 334: 438, 1988). These observations prompted us to question whether H⁺-ATPases are present in the plasma membrane of naturally occurring human tumor cells. In the present study, we screened two normal and 14 primary human tumorigenic cell lines for the presence of plasmalemmal H⁺-ATPase. Our results indicate that most tumorigenic cells maintain a higher pH\text{in}, and a higher rate of glycolysis, compared to non-tumorigenic cells. Bafilomycin, an inhibitor of H⁺-ATPase normally found in endosomes (V-type), induces a significant decrease in pH\text{in} in five tumor cell lines, but not in other tumor or normal cells. In contrast, SCH28080, an inhibitor of H⁺/K⁺-ATPase, did not affect pH\text{in} in any of these cells. Simultaneous measurement of pH in cytoplasm and endosomes-lysosomes indicates that
bafilomycin can differentially affect the pH of both of these compartments. This bafilomycin-induced cytosolic acidification at the endosomal-lysosomal level is probably not caused by inhibition of H\(^+\)-ATPases at the endosomal-lysosomal level, since one tumor cell line exhibited endosomal-lysosomal alkalinization with no changes in cytosolic pH. Moreover, mesothelioma cells exhibited a cytosolic acidification with no change in endosomal-lysosomal pH. These results suggest that a vacuolar type H\(^+\)-ATPase is expressed in the plasma membrane of some tumor cells and may contribute to their high resting pH\(^{\text{in}}\). Combined with the yeast H\(^+\)-ATPase studies, these results indicate that expression of V-type H\(^+\)-ATPases in the plasma membrane may play a role in the mechanisms of carcinogenesis.
INTRODUCTION.

Several previous investigations indicate that intracellular pH (pH\textsubscript{in}) changes may occur in response to mitogenic activation. These changes are complex and may involve several different H\textsuperscript{+} transporting mechanisms. There are at least three main systems involved in the regulation of pH\textsubscript{in} in eukaryotic cells: (a) mitogen-stimulated Na\textsuperscript{+}/H\textsuperscript{+} exchangers, which electroneutrally raise pH\textsubscript{in} and can be inhibited by amiloride and its derivatives (19, 31); (b) Na\textsuperscript{+}-dependent or -independent HCO\textsubscript{3}\textsuperscript{-} transporters which alkalinize or acidify the cytosol, respectively, and can be inhibited by stilbene disulfonate derivatives (4, 8); and (c) plasma membrane H\textsuperscript{+}-ATPases, which represent the least understood mechanism of pH\textsubscript{in} regulation (5, 8, 29). Understanding the role of H\textsuperscript{+}-ATPases in pH\textsubscript{in} regulation is complicated by their diversity. There are at least three types: a mitochondrial (F\textsubscript{T}/F\textsubscript{T}) ATPase (F-type), a vacuolar H\textsuperscript{+}-ATPase (V-type) and a plasma-membrane H\textsuperscript{+}/K\textsuperscript{+}-ATPase (P-type) (3, 39, 44, 46).

Recently, Perona and Serrano (40) have shown that tumorigenic transformation can be induced by transfection of NIH-3T3 cells with gene pma-1, which codes for yeast plasma membrane H\textsuperscript{+}-ATPase. We have shown that the pH\textsubscript{in} of cells transfected with the yeast H\textsuperscript{+}-ATPase is higher than their non-tumorigenic counterparts under physiological conditions (14), supporting the idea that a high
pH\textsuperscript{in} in vivo might be important to the tumorigenic phenotype. We hypothesize that mammalian cells which become tumorigenic may express H\textsuperscript{+}-ATPase on their cell surface and use it to maintain a high pH\textsuperscript{in}.

Evidence for the presence of H\textsuperscript{+} pumps in the plasma membrane of a tumor cell such as Ehrlich Ascites Tumor (EAT) cells has already been presented in the literature (22, 26, 44). Furthermore, H\textsuperscript{+}-ATPases have been observed in the plasma membranes of several types of normal cells including: hepatocytes (9), macrophages (46), osteoclasts (3, 50), epithelial cells (48), Chinese Hamster ovary (CHO) cells (26), parietal cells (50), and secretory cells from the urinary tract (49). In most of these cases, the H\textsuperscript{+}-ATPase involved is likely to be the P-type H\textsuperscript{+}/K\textsuperscript{+}-ATPase. However, in macrophages and osteoclasts, the H\textsuperscript{+}-ATPase appears to be a V-type (3, 46, 50). Therefore, there is no simple correlation between the presence of H\textsuperscript{+}-ATPase in the plasma membrane and tumorigenesis.

V-type H\textsuperscript{+}-ATPases are constitutively present in various endomembrane systems, including secretory vesicles, clathrin coated vesicles, synaptosomes, Golgi, endosomes and lysosomes (6, 8, 10, 29, 33-35, 44). It is believed that the V-type H\textsuperscript{+}-ATPases play a significant role in the maintenance of the acidic environment found in endosomes and lysosomes, i.e. early endosomes have a pH of ca. 6.5, late endosomes a pH of ca. 6, and lysosomes a pH of ca. 4.5-5.0 (10, 29, 38).
It is hypothesized that exposure of the material that is internalized by endocytosis to this decreasing pH is important in maintaining the orderly traffic and processing of receptors and ligands. It is therefore feasible that V-type H⁺-ATPases may be present on the plasma membrane of most eukaryotic cells, due to normal membrane-recycling mechanisms. It has been argued that this must be the case, since H⁺-ATPases are observed in clathrin-coated vesicles, which are derived from plasma membrane (E. Racker, personal communication). In some cases, alterations in the normal membrane-recycling mechanisms and/or other phenomena may lead to functional overexpression of the H⁺ pumps at the plasma membrane. We hypothesize that this overexpression can contribute to a tumorigenic phenotype.

At the present time, one of the most effective means of differentiating the V-ATPase from the F- and P-ATPases is provided by bafilomycins, a family of macrolide antibiotics. Vacuolar H⁺-ATPases are highly sensitive to Bafilomycin A₁, whereas P- and F-ATPases are moderately sensitive or insensitive to this drug, respectively (6). Binding of bafilomycin to the V-type H⁺-ATPase is irreversible and not competitive with ATP. Although the stoichiometry of binding to the H⁺-ATPase has not been unequivocally established, a stoichiometry of 1:1 has been estimated. IC₅₀ values of < 0.5 x 10⁻³ μmol bafilomycin/mg of protein have been found in vacuoles, chromaffin granules, neurosecretory granules, synaptosomes,
and macrophage plasma membrane (6, 33, 34, 46). One of the most selective inhibitors for the H⁺/K⁺-ATPase is SCH 28080 (30). It has been shown to inhibit H⁺/K⁺-ATPase by competing with K⁺ for binding (at the extracytosolic face) to E₂P and blocking K⁺ induced dephosphorylation. The apparent $K_\text{d}$ for SCH 28080 binding to H⁺/K⁺-ATPase increases from ca. 0.02 µM in the absence of K⁺ and acidic pH (5.5) to ca. 1.5 µM in the presence of 25 mM K⁺ and high pH (7.5) (30, 51).

There also are relatively non-specific H⁺-ATPase inhibitors such as N-ethylmaleimide, fusidic acid and suramin (32, 44). We have recently shown that it is possible to partially discriminate tumorigenic (RN1a and Ehrlich Ascites tumor cells) from non-tumorigenic cells (Chinese Hamster ovary cells and BALB/c) on the basis of the effects of these drugs on the pH<sup>in</sup> in these cells (26). In the present study, we have expanded on this previous work by employing the more specific H⁺-ATPase inhibitors, as well as, by screening several additional cell lines including both non-tumor and tumor murine and primary human cell lines. Furthermore, we have devised a technique which allows for the simultaneous measurement of pH in the cytosolic (pH<sup>cyt</sup>) and endosomal/lysosomal compartments (pH<sup>vac</sup>) in whole cell populations. This provides a method for discrimination of the specific site of action of the H⁺-ATPase inhibitors, i.e. plasma membrane versus endosomal/lysosomal membrane.
MATERIAL AND METHODS

Cell Culture. NIH-3T3 mouse embryo cells were provided by Dr. Albert Liebowitz (Cancer Center, University of Arizona). These cells were cultured as described in Gillies et al. (12). Briefly, the cells were cultured in Dulbecco's modified Eagle media (DMEM-Gibco, Grand Island, NY), supplemented with 10% Nu-serum (Collaborative Research, Bedford MA), in a 5% CO₂ atmosphere at 37°C. Cells were grown in T-75 culture flasks and passed bi-weekly at an inoculation density of 2 x 10⁵ cells per 75 cm² flask. For fluorescence measurement using pyranine as a pHₗₙ indicator, cells were inoculated into 90 mm petri dishes at a density of 2 x 10⁵ in DMEM plus 10% Nu-serum. Two days thereafter, these cultures were prepared for pHₗₙ measurements by fluorescence as described below. For fluorescence measurements using SNARF-1, and for simultaneous measurements of pH in cytosolic and vacuolar compartments, cells were inoculated at 5 x 10⁴ cells/60 mm petri dish containing glass coverslips (9 x 22 mm).

RN1a cells are derived from NIH-3T3 cells transfected with yeast pma-1, which codes for plasma membrane H⁺-ATPase (40). These cells were provided by Dr. Rosario Perona (Instituto de Investigaciones Biomedicas, Madrid, Spain). These cells were cultured in high glucose DMEM, supplemented with 10% Nu-serum. Culture conditions for experiments were performed as described for NIH-3T3 cells.
and subcultured for experiments in a likewise manner.

N-MUT cells are also derived from NIH-3T3 cells transfected with a less active form (allele PMA-213) of the yeast H⁺-ATPase (41). These cells have 40% less ATPase activity and 20% less H⁺-pumping activity than RN1a cells. These cells were also provided by Dr. Perona and grown under identical conditions as the parental NIH-3T3 cells.

Primary human non-tumor and tumorigenic cells provided by Dr. Albert Leibowitz (Arizona Cancer Center), as well as, established cell lines (from American Type culture Collection) were used. Human primary cells included: non-tumor fibroblasts from esophagus and human foreskin; primary tumor cells [breast (from two different individuals, i.e. breast-I and breast-II), Ewing's sarcoma, giant cells, glioblastoma, leiomyosarcoma, melanoma (from two different individuals, i.e. melanoma-I and melanoma-II), mesothelioma, and carcinomas from lung, ovary, pancreas, and uterus]. Cells were obtained from adult volunteers undergoing biopsy and/or surgery for either diagnosis or as a part of their therapy, respectively. They were grown out as explants from 1 g of tissue in medium M15 (Sigma Co.) plus 10% Fetal bovine serum (Hyclone), supplemented with (in g/L): reduced glutathione (0.015); catalase (0.005); methyl cellulose 15 CPS (2.0); polyvinylpyrrolidone-360 (1.0); sodium selenite (1.73 x 10⁻⁶); 2 mercaptoethanol
(0.0008); orotic acid (0.015); and DL-ornithine (0.015). After the initial inoculum, cells were grown for 7-15 passages in this medium, and subsequently grown for experiments essentially as described for RN1a cells. Cells used in this study were in passage 24-45.

Established human cell lines obtained from American Type Culture Collection that included breast cancer (MDA-MB-231); and lung carcinoma (A549) cell lines were also used, and were grown as described for RN1a cells.

Buffers. Cell Suspension Buffer (CSB) contained: 1.3 mM CaCl$_2$, 1 mM MgSO$_4$, 5.4 mM KCl, 0.44 mM KH$_2$PO$_4$, 110 mM NaCl, 0.35 mM NaH$_2$PO$_4$, 5 mM glucose, 2 mM glutamine and 25 mM HEPES, at a pH of 7.15 at 37°C. High K$^+$ buffer. (High K$^+$) contained: 5 mM glucose, 2 mM glutamine, 10 mM HEPES, 10 mM MES, 10 mM Bicine and the corresponding intracellular K$^+$ concentration for each cell line (26).

Culture Preparation for SNARF-1 measurements. Subconfluent cell cultures grown onto coverslips were used for most experiments. Cells were loaded with the pH indicator SNARF-1 using the cell permeant acetoxyethyl ester (AM) form as described fully in Martinez-Zaguilán, et al. (27). Briefly, coverslips containing cells were washed three times with CSB. Subsequently, two coverslips were incubated
for 30 min at 37°C, in a 5% CO₂ atmosphere with 3 ml of CSB containing 7 μM SNARF-1 (AM). Cellular esterases cleave the ester groups of the acetoxymethyl ester form to yield the free acid which is more impermeant, and therefore, "trapped" within cells. After the 30 min incubation, buffer was removed by aspiration and the cells were washed three times with CSB containing 0.2% (v/v) Nu-serum (0.2% CSB), followed by a second incubation in 0.2% CSB for 30 minutes, to allow for complete hydrolysis of the dye. After this time, two coverslips were placed back to back in a holder/perfusion device (38), which was subsequently inserted into the fluorometer cuvette. Sample temperature was maintained at 37°C by keeping both the water jacket and the perfusion buffer at 37°C using a circulator water bath (Lauda Mod. RM20, Brinkmann Instruments Co.).

Culture Preparation for Pyranine Measurements. Intracellular pH was also determined from the fluorescence of pyranine as described in Giuliano and Gillies (15). Briefly, cells plated onto 90 mm petri dishes cultures were washed three times with CSB at pH 7.15, scrape-loaded with 1 mM pyranine, washed by centrifugation at 400 xg during 5 min at 10°C, resuspended in the same buffer and incubated at 37°C for 1 hr, to allow the cells to recover. After the recovery period, cells were washed by centrifugation and the pellet resuspended in CSB at 2 x 10⁶/3 ml in the fluorometer cuvette for pH⁶n measurements. Fluorescence
measurements were performed in a temperature-controlled continuously stirred cuvette.

Culture preparation for Simultaneous Measurement of pH in Cytosolic (pH<sub>cyt</sub>) and endosomal/lysosomal (pH<sub>vac</sub>) Compartments. For the measurement of pH<sub>cyt</sub> and pH<sub>vac</sub> we took advantage of the fact that 7-hydroxy Coumarin conjugated to dextran (Coumarin-DEX) (70,000 M.W.) has a relatively low pK<sub>a</sub> and is membrane impermeant but can be taken up by the cells by endocytosis. Once inside of the cells, this dye is not cytotoxic (data not shown). Therefore, cells were plated onto coverslips and, after 24 hrs in culture, the medium was exchanged by medium containing 5 µg/ml Coumarin-DEX. After 3-24 hrs in the presence of Coumarin-DEX, the dye is mainly localized into acidic compartments, and therefore can be used to measure pH<sub>vac</sub>. For our experiments we use 12-16 hrs to load cells with Coumarin-DEX, after which time cells are loaded with SNARF-1(AM), for measurement of pH<sub>cyt</sub>. In these experiments, cells are incubated with SNARF-1 (7 µM) for an additional 30 min, at 37°C. Subsequent handling of these cells is as described above for SNARF-1 measurements. Confirmation of the exact subcellular localization of each indicator was performed using fluorescence microscopy of single cells (see Fig 8).

pH Measurements. Fluorescence Measurements were carried out in a
temperature-controlled unit housed in an SLM8000C (SLM. Urbana, ILL), at 37°C using 4 nm slits and an external rhodamine standard as a reference. For pH\textsuperscript{ln} measurements using SNARF-1, data were collected in a continuous acquisition mode, where excitation was set at 534 nm and emission sequentially sampled at 584, 600 and 644 nm. A single cycle was completed in 3.68 seconds. The fluorescence at 600 nm represents the isoemission point for SNARF-1 (27), whereas the fluorescence at 584 and 644 nm are pH sensitive. Data were translated to ASCII format. The ratio of sequential fluorescence intensities of emissions at 584 and 644 nm (R) was converted to pH\textsuperscript{ln} values using eqn. [1].

\[ \text{pH} = \text{pK}_a + \log \left( \frac{S_{f2}}{S_{b2}} \right) + \log \left[ \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \right] \] eqn. [1]

where \( R_{\text{min}} \) represents the ratio for the fully protonated dye; \( R_{\text{max}} \) the ratio for the fully de-protonated dye; \( R \) is the ratio at any given pH, \( R \) values increases with increasing pH; \( S_{f2} \) and \( S_{b2} \) are the fluorescence values for the free and bound forms of the dye, at the denominator wavelength, and are used to correct for the fact that the denominator wavelength is ion-sensitive.

The fluorescence of pyranine was also used to monitor cytoplasmic pH of cells in suspension. Although this dye is membrane-impermeant it can be internalized by scrape-loading (28). For pH\textsuperscript{ln} measurements using pyranine, data were
collected as described for SNARF-1, except the dye was excited at 402, 415 and 465 nm and its emission was collected at 514 nm. This cycle lasted 3.33 sec. and was continuously repeated to generate an experimental time course. The fluorescence at 415 nm corresponds to the isoexcitation point for this dye and can be used to evaluate the efficiency of dye-loading, and/or quenching artifacts. pH<sup>i</sup> was determined by 465/402 fluorescence excitation ratio using eqn [1].

Selective loading of the cytoplasmic (SNARF-1), and the endosomal/lysosomal compartments (Coumarin-DEX), provides a precise method to simultaneously measure pH<sup>cyt</sup> and pH<sup>vac</sup>, respectively. An important feature of Coumarin-DEX is that, in analogy to SNARF-1, it can report pH using ratio spectroscopy. In addition, the respective isoexcitation and isoemissive points for Coumarin-DEX and SNARF-1, offer further advantages, not only to quantify dye concentration and efficiency of dye loading, but also to check for possible artifacts such as quenching and leakage. For simultaneous measurement of pH<sup>cyt</sup> and pH<sup>vac</sup>, the acquisition parameters were set as follows: excitations at 352, 370, and 402 collecting emissions at 448 nm (Coumarin-DEX conditions); followed by excitation at 534 nm, collecting emissions at 584, 600, and 644 nm. A single cycle was completed in 13.3 seconds. Conversion of ratio values to pH<sup>cyt</sup>/pH<sup>vac</sup> were performed as described above, by using equation [1].
In vitro pH values used for calibration were obtained with a Beckman model 71 pH meter, using a Corning glass combination electrode. The electrode was calibrated at two known temperature controlled pH values using commercially prepared standards from VWR Scientific (San Francisco CA).

**In Situ Calibration of SNARF-1 and Pyranine.** The calibration of pH dyes was performed as described previously (14, 27). Briefly, attached (SNARF-1 loaded) cells were perfused with their corresponding high K+ buffer plus 6.8 μM of the K+/H+ ionophore nigericin and 2 μM valinomycin, which elicits equilibration of intra- and extracellular pH; i.e., pH$^{\text{in}}$ of the cells is equal to pH$^{\text{ex}}$. For these experiments, pH$^{\text{ex}}$ was varied from pH 6.0 to 8.0. Suspension (pyranine-loaded) cells were resuspended in high K+ buffer at various known pH$^{\text{ex}}$ values ranging from 5 to 8, and their spectra were recorded. From these in situ calibration curves one can obtain R values at each pH$^{\text{ex}}$ studied. Equation [1] is then solved iteratively using non-linear analysis (MINSQ, MicroMath Scientific Software, Salt Lake City, Utah), yielding values of $R_{\text{max}}$, $R_{\text{min}}$ and $pK_a$ for the dye. Such calibration was carried out for each of the cell types (c.f. 27). From these in situ calibration curves, the following parameters were estimated for pyranine: $pK_a = 7.407 \pm 0.0817$; $R_{\text{min}} = 0.0651 \pm 0.0271$, $R_{\text{max}} = 2.052 \pm 0.125$; $S_{\text{r2}}/S_{\text{c2}} = 0.745 \pm 0.081$. For SNARF-1, the following parameters were estimated: $pK_a=$
7.378 $\pm$ 0.062; $R_{\min}= 0.458 \pm 0.120$; $R_{\max}= 1.928 \pm 0.115$; $S_{S_1}/S_{S_2}= 0.822 \pm 0.062$. In situ calibration of each dye has been performed in 7 out of 12 cell lines. Under our experimental conditions these calibration parameters are comparable within 10% error among the different cell lines. In order to normalize pH$_{\text{ln}}$ calculations between the various cell types, mean values for each calibration parameters were computed from the in situ calibration data collected for all cells studied. Each of those cell lines were in situ calibrated using at least 15 different pH values. Similar in situ calibration parameters have been obtained in a variety of other cell lines, and have been reported elsewhere (14, 27).

pH$_{\text{vac}}$ was estimated from in vitro calibration curves and not from in situ calibration parameters due to the uncertainty of equilibration of extracellular K$^+$ with the lysosomal/endosomal compartments. From such in vitro calibration curves, the following parameters were estimated: $R_{\min}= 0.0099 \pm 0.002$; $R_{\max}= 6.054 \pm 0.019$; and $pK_a = 6.701 \pm 0.019$.

**Cell Number.** Cell number was determined using a dye binding assay, as described by Gillies et al. (11). Briefly, cells were plated at $3 \times 10^4$ in 24-well plates (Flow Labs), and 12 hours thereafter various concentrations ($1 \text{nM} - 1 \text{mM}$) of bafilomycin and SCH 28080 were added. At 24, 48 and 72 hours following
addition of H⁺-ATPase inhibitors, the medium was removed, and the cells were washed with CSB at 37°C. This procedure allows one to stain only viable cells that remained attached to the plastic well. Subsequently, the cells were fixed with 1% glutaraldehyde, and then stained with 0.1% crystal violet. Subsequently, the dye was solubilized in 0.2% Triton X-100. The absorbance at 590 nm of this Triton X-100 solution is linearly related to the number of viable cells (11).

**Colony Formation.** The capability of tumor and non tumor cells to form colonies was assessed as described previously (20). Briefly, an underlayer of 0.5% Bacto agar (DIFCO) in DMEM medium, containing 20% Nu-serum was prepared (1 ml/35 mm Falcon plastic Petri dish). Subsequently, cells were suspended in a plating layer (1 ml) of 0.3% agar in DMEM plus 20% Nu-serum at a final cell density of 4 x 10⁴. After two weeks in culture, with no additional feeding, cells were stained with 1.97 mM p-iodonitrotetrazolium violet for 48 hrs. Colonies were counted using an automated colony counter that discriminates colonies in terms of their diameter (Fas-2 Omnicon image counter, Bausch and Lomb). For our experiments only colonies of 60 μm of diameter or greater were considered.

**Glycolytic rate.** Cells were plated into 24 mini-wells at 3 x 10⁴, and the glucose and lactate concentrations in the media were monitored periodically for up to 96 hrs using an automated glucose/lactate analyzer (Yellow Springs Instruments
Model 2000). Cell number was also monitored as described above (11).

**Chemicals and Drugs.** Bafilomycin was generously provided by Dr. Altendorf (Universität Osnabrück, Federal Republic of Germany), and was prepared as a sterile stock solution of 1 mM in DMSO, protected from the light and stored at -20°C, until used. SCH 28080 was provided by Dr. A. Barnett (Schering Corporation, Bloomfield, NJ), and was prepared as described for bafilomycin. SNARF-1, Coumarin-DEX, and pyranine were obtained from Molecular Probes (Eugene, OR). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical.
RESULTS.

Establishment of cell lines: colony formation correlates with tumorigenicity.

The capability of cells to form colonies in soft agar generally correlates with tumorigenicity (20). The competence of the cells used in this study to form colonies in agar was therefore evaluated. Figure 1 shows that non tumor cells (NIH, N-MUT and normal esophageal cells) do not form colonies. As expected, all tumor cells (i.e. murine or human) are capable of forming colonies in 20% serum (Fig 1). This ability of the tumor cells to form colonies was also observed at 0.2% serum, although the number of colonies was decreased by 60 to 80% (data not shown).

Tumor cells maintain a high pH\textsuperscript{in}.

The present study was undertaken to ask whether or not a H\textsuperscript{+}-ATPase(s) is(are) present in naturally occurring tumor cells. As shown in figure 2, the measurement of pH\textsuperscript{in} using the fluorescence of SNARF-1 in tumor and non-tumor cells grown onto coverslips demonstrates that all but one of these tumor cells exhibit a higher
pH_in than non-tumor cells, i.e. NIH, N-MUT, and normal esophageal cells. The only exception was found in giant cells, where the pH_in is similar than that determined in non tumor cells. These measurements of pH_in were performed at a pH_ex = 7.15, in HCO_3^- free CSB and the absence of serum. Under these conditions, the activities of Cl^-/HCO_3^- exchange and Na^+/H^+ exchange are reduced by the absence of HCO_3^- and serum, respectively. Since the Na^+/H^+ exchange is supposed to be inactive above a pH_in of 7.25 (for a review, see 18, 31) cells with a steady state pH_in > 7.25 must have an alternate H^+ exporting mechanism, such as an H^+ -ATPase.

Tumor cells have a high rate of lactic acid production.

NIH 3T3 cells transformed with the yeast pma-1 gene, which codes for plasma membrane H^+-ATPase, glycolyze 5-10 times more rapidly than their non transformed counterparts (14). As a measure of the metabolic status of the cells used in this study, we have monitored glucose consumption and lactic acid production in both tumor and non tumor cells and have found that the glycolytic rate is substantially higher in tumor cells. Accordingly, the rate of lactic acid production in the tumor cells exceeds that exhibited by non-tumor cells (Fig 3). The only exception to this generalization was found in the giant cells, where the
rate of lactic acid production is similar to that observed in non-tumor cells. The specific lactic acid production values were derived from lactic acid measurements performed over a period of 72 hrs, at intervals of 24 hrs, after correction for cell growth. This correction for cell growth was made within the same experiment by monitoring cell number as described by Gillies et al. (11). During this period of time, the specific rate of glucose consumption and lactic acid production is linear (data not shown). We have recently shown that high glycolytic rates are not caused by the high pH\textsuperscript{in} \textit{per se}, and therefore reflect another property of these cells, such as increased ATPase activity (14).
FIGURE 1. Colony formation in tumor and non-tumor cells. Cells were plated in 3% agar plates, at 4 x 10⁴/35 mm Petri dish in DMEM + 20% Nu-serum (see Materials and Methods). After two weeks in culture, with no additional feeding, cells were stained with 1.97 mM p-iodonitrotetrazolium violet for 48 hrs under culture conditions. Colonies were counted using an automated colony counter. Only colonies greater than 60 μm in diameter are shown.
FIGURE 2. Mean pH\textsuperscript{in} for various non-tumor and tumor cell populations. Cells were grown on coverslips and loaded with SNARF-1 were used for determination of pH\textsuperscript{in} (see Materials and Methods). Cells were continuously perfused with CSB, pH\textsuperscript{ex} = 7.15 and resting pH\textsuperscript{in} was calculated from the ratio of fluorescence intensities at emission wavelengths of 644 and 584 nm (kex = 534 nm) using eqn [1].
FIGURE 3. Rate of lactic acid production in various non-tumor and tumor cell populations. Tumor and non tumor cells were grown as described in Materials and Methods and plated at 1 x 10^4 into 24 miniwell-plates. At various time periods (i.e. 0, 24, 48 and 72 hrs), the media was collected and lactate was determined by using a simultaneous glucose/lactate analyzer. At the corresponding time points, cells were fixed and stained with crystal violet to monitor cell number (11). The values represent the mean ± S.E. from 3 experiments (see Materials and Methods).
Bafilomycin (a $\text{H}^+$-ATPase inhibitor) but not SCH 28080 (a $\text{H}^+/\text{K}^+$-ATPase inhibitor), decreases $\text{pH}^\text{in}$ in tumor cells.

We have screened for the presence of $\text{H}^+$-ATPases in both tumorigenic and non-tumorigenic cells by using a specific V-type $\text{H}^+$-ATPase inhibitor, Bafilomycin (6, 46), and an specific $\text{H}^+/\text{K}^+$-ATPase inhibitor, SCH 28080 (30). Figure 4 shows that addition of $1.0 \text{ M}$ bafilomycin to cells known to contain a $\text{H}^+$-ATPase in their plasma membrane such as RN1a cells, results in a decrease in $\text{pH}^\text{in}$ of ca. 0.22 pH units (Fig 4A). In contrast, addition of $1.0 \text{ M}$ bafilomycin to the parental cell line, NIH-3T3 induces only a minor decrease in $\text{pH}^\text{in}$ of ca. 0.04 pH units (Fig 4B).

Addition of $1.0 \text{ M}$ bafilomycin to human leiomyosarcoma cells results in a dramatic decrease in $\text{pH}^\text{in}$ from a resting $\text{pH}^\text{in} = 7.47$ to $\text{pH}^\text{in} = 7.36$. This effect is concentration dependent, since addition of $10 \text{ M}$ bafilomycin to these cells induces a further decrease in $\text{pH}^\text{in}$ to 7.14 (Fig 4C). In contrast, addition of $1.0 \text{ M}$ bafilomycin to normal human esophageal cells does not affect $\text{pH}^\text{in}$, and subsequent addition of $10 \text{ M}$ bafilomycin results in only a minor decrease in $\text{pH}^\text{in}$ of $< 0.03$ pH unit (Fig 4D). Similar experiments have been performed with a variety of tumor cell lines. These data are shown in Fig 5A, and illustrate that $1.0 \text{ M}$ bafilomycin decreases $\text{pH}^\text{in}$ in most cells, but that some cells are more strongly affected that others. We have titrated the effect of this drug on $\text{pH}^\text{in}$ in a variety of cell lines and have observed that the sensitivity and magnitude of the
pH<sub>in</sub> decrease is cell-type dependent. For instance, the pH<sub>in</sub> of RN1a, leiomyosarcoma, mesothelioma, breast carcinoma and a melanoma cell line, are all significantly decreased by bafilomycin, while the pH<sub>in</sub> of all other cells does not decrease by more than 0.05 pH unit. Although these effects were highly reproducible, it is likely that changes of pH<sub>in</sub> ≤ 0.05 pH unit are not significant in the present study. The acidification observed in the RN1a cells is likely due to inhibition of the yeast H<sup>+</sup>-ATPase by bafilomycin (D. Perlin, personal communication).
FIGURE 4. Time course of bafilomycin effects on pH\textsubscript{in} in tumor and non-tumor cells. A. RN1a cells; B. NIH-3T3 cells; C. Leiomyosarcoma cells; D. normal esophageal cells. Cells were plated onto coverslips and loaded with SNARF-1, as a pH\textsubscript{in} indicator, as described under Materials and Methods. Cells were perfused in the fluorometer cuvette with HCO\textsubscript{3}-free CSB, pH\textsubscript{ex} 7.15. At the times indicated, perfusate was exchanged for one containing the indicated concentration of bafilomycin.
We have also tested for the presence of H⁺/K⁺-ATPase in tumor and non tumor cells. Figure 6 shows that 1.0 μM SCH 28080 induces a pH\textsubscript{in} decrease of ca. 0.2 pH units in NIH-3T3 cells (Fig 6A). Similar results were observed in N-MUT cells (data not shown). Addition of 1 μM SCH 28080 to normal human esophageal cells results in a decrease in pH\textsubscript{in} of 0.04 pH units (Fig 6B). Neither of these cell lines are tumorigenic. In contrast, addition of SCH 28080 at concentrations as high as 50 or 100 μM did not affect pH\textsubscript{in} in the tumorigenic RN1a (Fig 6C), or leiomyosarcoma (Fig 6D) cells. No significant effect of SCH 28080 on pH\textsubscript{in} was observed in any of the other cells as shown in figure 5B.
FIGURE 5. Effect of bafilomycin (A) and SCH 28080 (B) on pH\textsuperscript{n} in tumor and non tumor cells. Handling of these cells was performed as described in legend to Fig 4. Δ pH\textsuperscript{n} was evaluated by determining the steady state pH\textsuperscript{n} before treatment and subtracting this from the pH\textsuperscript{n} after treatment, at the nadir (c.f. Fig 4 and Fig 6). Data represent the mean ± S.D. of 3-5 experiments per data point, and were obtained from a series of experiments similar to those shown in figures 4, 6, and 9 (see Materials and Methods).
FIGURE 6. Time course of SCH 28080 on pH in in tumor and non tumor cells. A. NIH-3T3 cells; B. normal esophageal cells; C. RN1a cells; D. Leiomyosarcoma cells. Cells were handled as described in legend of Fig 4. At the times indicated, perfusate was exchange for one containing the indicated concentrations of SCH 28080.
Simultaneous measurement of intracellular pH ($\text{pH}^{\text{in}}$) and endosomal/lysosomal pH ($\text{pH}^{\text{vac}}$).

The data presented above suggest that some tumor cells express a bafilomycin sensitive (e.g. V-type) H$^+$-ATPase in their plasma membrane. This same enzyme contributes to the maintenance of the acidic interior of lysosomes and endosomal vacuoles (10, 29, 38). Therefore, the possibility exists that bafilomycin might have specifically inhibited a H$^+$-ATPase at the level of the endosome/lysosome if it permeates the cell membrane. Inhibition of such a system may result in acidification of the cytosol, associated with an alkalinization of the endosomal/lysosomal compartment. Therefore, in order to accurately evaluate our findings it was critical to demonstrate that inhibition of the H$^+$-ATPase occurs at the plasma membrane level, and not at the vacuolar level. We examined this by simultaneously measuring the endosomal/lysosomal and cytoplasmic pH using the fluorescence of Coumarin-DEX and SNARF-1, respectively. A primary assumption of this simultaneous measurement is that there is no fluorescence energy transfer between the two fluoroprobes. Fluorescence energy transfer results primarily from dipole-dipole interactions between fluorophores (i.e. the donor and the acceptor), and is therefore affected by both the relative orientation and the relative distance between them (usually < 100 Å) (25). Furthermore,
energy transfer is maximal when the emission spectrum of one fluoroprobe overlaps the excitation spectrum of the other. Figure 7 illustrates that there is little spectral overlap between the excitation and emission spectra of these probes, suggesting that there would be little or no energy transfer between SNARF-1 and DEX-Coumarin. We have also measured both SNARF-1 and Coumarin-DEX fluorescence and have determined that the presence of the other dye has no effect on the quantum yield, the $pK_a$, $R_{min}$ nor $R_{max}$ of these two pH indicators (data not shown). Therefore, accurate simultaneous measurement of $pH_{cyt}$ and $pH_{vac}$ can be made using Coumarin-DEX and SNARF-1.

An additional problem in simultaneous measurement of pH in two different compartments within the same cell is the specific localization of each dye. Fortunately, the cytoplasmic and endosomal/lysosomal compartments differ dramatically in terms of their intrinsic pH, and therefore, it is possible to assess the distribution of these dyes based on their fluorescence spectra. We also utilized forms of the dyes which differed in their membrane-permeability. Coumarin-DEX is a membrane-impermeable compound with a high molecular weight, and consequently should be taken up by the cells by endocytosis and hence trapped into acidic compartments. On the other hand, SNARF-1(AM) is a membrane permeable dye and is trapped into the cytosol upon cleavage of the acetoxymethyl ester form by non-specific esterases. However, the ability of
SNARF-1 (AM) and deesterified SNARF-1 to localize within the acidic compartment is unclear. The respective subcellular distributions of the dyes were directly analyzed using digital imaging microscopy of single cells. As shown in figure 8, SNARF-1 is uniformly distributed in the cytoplasm of NIH-3T3 cells, whereas Coumarin-DEX is compartmentalized into endosomes/lysosomes (cf. Fig 8A, 8B). Coumarin-DEX (to label endosomes/lysosomes) was also co-loaded with vital dyes to specific subcellular organelles. Figure 8C shows a cell co-loaded with Coumarin-DEX and rhodamine-123 to label mitochondria. Clearly, Coumarin-DEX and rhodamine-123 are in distinct subcellular compartments. These data support our contention that Coumarin-DEX compartmentalizes into endosomes/lysosomes and not in other organelles.
FIGURE 7. Fluorescence spectra of SNARF-1 and Coumarin-DEX. 2 μM SNARF-1 and 5 μg/ml of 7-hydroxy Coumarin conjugated to dextran (70,000 M.W.) were dissolved, separately, in a modified Hank's buffer and their fluorescence emission or excitation spectra were recorded at various pH's. The prominent features of the Coumarin-DEX spectra are the peaks at excitations of 352 nm and 402 nm, respectively which decrease and increase as pH is increased, associated with no changes in fluorescence at 370 nm (isoexcitation). For SNARF-1, the prominent features of the spectra include the peaks at emissions of 584 nm and 644 nm which respectively decrease and increase as pH is increased, with no changes in fluorescence at 600 nm (isoemissive).
FIGURE 8. Subcellular distribution of SNARF-1 (A) and Coumarin-DEX (B) in NIH-3T3 cells. Cells were co-loaded with Coumarin-DEX and SNARF-1 as described under Materials and Methods. Fluorescent images of cells were obtained using a Photometrics CH250 CCD camera attached to an inverted microscope (Olympus) equipped for epifluorescence. A 60 X Olympus UV-fluor objective was used for cell imaging and a 150-W xenon arc lamp was used as the excitation source. SNARF-1 was excited at 480 nm using a 20 nm bandpass filter (Omega) and emission was collected at 535 nm (A). Coumarin was excited at 350 nm using a 20 nm bandpass (Omega), and emission was collected at 450 nm (B). (C) Cells were co-loaded with Coumarin-DEX and rhodamine 123 to label mitochondria. Rhodamine-123 was excited at 475 using a 10 nm bandpass (Omega) and emission was collected at 525 nm. Digitized images were analyzed using a Silicon Graphics Personal Iris. Bar = 10 μm.
Simultaneous measurement of $pH_{in}$ and $pH_{vac}$ indicates that bafilomycin interacts with plasma membrane $H^+\text{-ATPases}$ in tumor cells.

Figure 9 illustrates simultaneous measurement of $pH_{cyt}$ and $pH_{vac}$ in human tumor and non tumor cells. Notice the differences in resting $pH_{cyt}$ and $pH_{vac}$, which corresponds to the expected pH values in these compartments, i.e. $> 7.0$ and $< 6.5$, respectively. This endosomal/lysosomal pH is slightly higher than expected, and may reflect some dye leakage into the cytosol or a mean measure of acidic and less differentiated endosomes. However, the signal remains stable for longer periods of time, indicating that the dye is evenly distributed in a single "acidic" compartment. If the dye were consistently leaking out to the cytoplasm, then one would expect to see an increase in fluorescence ratio, and eventually would report $pH_{vac}$ equal to $pH_{cyt}$. As shown in figures 9 and 10, this is not the case. Therefore we must assume that the dye is trapped into the endosome/lysosome compartment. A test of the utility of Coumarin-DEX and SNARF-1 to measure endosomal/lysosomal and cytosolic pH independently was performed by acid loading experiments. Incubation of cells with $NH_4\text{Cl}$ results in elevation of $NH_4$ due to free diffusion of $NH_3$ across the plasmalemma. As shown in Fig 10, $NH_4\text{Cl}$ treatment resulted in simultaneous increase in $pH_{cyt}$ and $pH_{vac}$ in NIH-3T3 cells that is due to the entrance of the uncharged $NH_3$ specie, which rapidly crosses both the plasma and the lysosomal membranes, raising $pH_{cyt}$ and
pH$_{\text{vac}}$. Removal of NH$_4$Cl resulted in acidification followed by pH$_{\text{cyt}}$ and pH$_{\text{vac}}$ recovery with different kinetics. This provides further support for the assertion that the dyes are located within different compartments.

Perfusion of cells with 1 µM bafilomycin resulted in a variety of cell-dependent responses (Fig 9). Human foreskin fibroblasts (normal) are not affected by bafilomycin, indicating that there are not V-type H$^+$-ATPases at the plasma membrane, or that the drug is not crossing the plasma membrane, and therefore, does not interact with H$^+$-ATPases at the vacuole (c.f. Fig 9A). Bafilomycin treatment resulted in an alkalinization in endosome/lysosomal compartments in breast carcinoma cells (Fig. 9B). In contrast, lung carcinoma cells responded with a decrease in pH$_{\text{cyt}}$ and an increase of pH$_{\text{vac}}$, indicating an effect of bafilomycin at both the plasma membrane and at the endosome/lysosome membrane (Fig 9D). Addition of bafilomycin to mesothelioma cells resulted in a dramatic decrease in pH$_{\text{cyt}}$ with only slight changes in pH$_{\text{vac}}$, suggesting that bafilomycin is interacting at the plasma membrane and not the vacuole. In parallel experiments, we also observed that addition of 10 µM bafilomycin to mesothelioma cells results in a large decrease of ca. 0.4 pH unit at the cytoplasmic level, associated with a minor increase in endosomal/lysosomal pH (data not shown). This apparently high concentration of bafilomycin would correspond to approximately 0.01 µmol/mg of cell protein, a concentration that does not affect the activity of F- or P-ATPases,
but inhibits V-ATPases with high specificity (6). We also tested for the effect of bafilomycin on F-ATPase by monitoring oxygen consumption and found no effect at concentrations as high as 10 μM (data not shown). These results support our contention that the decrease in pH\text{\textsuperscript{ln}} observed upon bafilomycin treatment in mesothelioma cells is due to inhibition of a V- H\textsuperscript{+}-ATPase located at the plasma membrane and not at the acidic vacuole or by inhibition of respiration (F type H\textsuperscript{+}-ATPases).

During the course of these simultaneous pH\text{\textsuperscript{cyt}} and pH\text{\textsuperscript{vac}} measurements, we observed that pH\text{\textsuperscript{vac}} values found in tumor cells are at least 0.3 - 1.0 pH units higher than in non-tumor cells. This could be the result of a real difference in pH\text{\textsuperscript{vac}} or may reflect differences in the ratio and/or proportion of endosome/lysosome between tumor and non-tumor cells. Suggestions of a high lysosomal pH has been observed upon transformation of murine and human fibroblasts with either v-K-ras or T24 H-ras oncogenes, respectively (24).

The effect of bafilomycin and SCH 28080 on cell growth in non tumor and tumor cells was also evaluated. Our results indicated that SCH 28080 does not affect cell growth for up to 72 hrs, in a range of concentrations from 10 nM to 100 μM (data not shown). In contrast, bafilomycin suppresses cell growth in both non tumor and tumor cells. Within the first 24 hrs < 50% inhibition of cell growth was
achieved at the concentrations used in this study. Complete suppression of cell growth was observed at 48 and 72 hrs. The calculated $K_i$'s at 48 and 72 hrs were similar for all cell lines (ranging from 80 to 150 $\mu$M). Thus, it is likely that at high concentrations, bafilomycin has effects on cell physiology distinct from its effects on plasmalemmal $V$-$H^+$-ATPase. It is important to note also, that the concentrations of bafilomycin used in the above pH studies were not toxic during the time frame of our pH measurements.
FIGURE 9. Effect of bafilomycin on cytoplasmic pH (pH$_{cyt}^*$) and endosomal/lysosomal pH (pH$_{vac}^*$). Cells were plated onto coverslips and were loaded with SNARF-1 and Coumarin-DEX (see Materials and Methods) to measure pH$_{cyt}^*$ and pH$_{vac}^*$, respectively. Cells were perfused with HCO$_3^-$-free CSB, pH$_{ext}^*$ 7.15. At the times indicated, the perfusate was exchanged for one containing 1 μM bafilomycin. HFSF = human foreskin fibroblasts.
FIGURE 10. NH₄Cl induces changes in both pH⁰⁰ and pH⁰ with different kinetics. NIH-3T3 cells were plated onto coverslips and were loaded with SNARF-1 and Coumarin-DEX (see Materials and Methods) to measure pH⁰⁰ and pH⁰, respectively. Cells were perfused with HCO₃⁻-free CSB, pH⁰⁰ 7.15. At minute 11, the perfusate was exchanged for one containing 50 mM NH₄Cl in HCO₃⁻-free CSB. At minute 30, the perfusate was exchanged for HCO₃⁻-free CSB.
Table I. Summary of Results

**EFFECT OF BAFILOMYCIN ON CYTOPLASMIC pH (pH<sup>cyt</sup>) AND ENDOSOMAL/LYSOSOMAL pH (pH<sup>vac</sup>)**

<table>
<thead>
<tr>
<th>NON-TUMORIGENIC CELLS</th>
<th>pH&lt;sup&gt;cyt&lt;/sup&gt;</th>
<th>pH&lt;sup&gt;vac&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human Foreskin Fibroblasts</td>
<td>-</td>
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</tr>
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</table>

**TUMOR CELLS**

<table>
<thead>
<tr>
<th></th>
<th>pH&lt;sup&gt;cyt&lt;/sup&gt;</th>
<th>pH&lt;sup&gt;vac&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast (I)</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>Breast (II)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Breast (Mâ¥239 ATCC)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ewing's sarcoma</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>Giant</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung (A549 ATCC)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melanoma (I)</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>Melanoma (II)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Mesothelioma</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ovarian</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uterus</td>
<td>-</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Data was obtained from at least 9 independent simultaneous pH<sup>cyt</sup> and pH<sup>vac</sup> measurement using the fluorescence of SNARF-1 and Coumarin-DEX. A minus sign indicates that no effect or < 0.05 pH unit change was observed upon 1 µM bafilomycin treatment. A plus sign indicates that a > 0.05 pH unit change was observed upon 1 µM bafilomycin treatment. N.D. = not measured.
DISCUSSION.

It has been suggested that changes in pH\textsuperscript{ln} are important for cell proliferation (23, 43). However, others have suggested that a high pH\textsuperscript{ln} plays only a permissive role on cell growth since pH\textsuperscript{ln} changes are not associated with mitogenesis \textit{in vitro} (31). Furthermore, we and others have shown that, upon mitogenic stimulation under cell culture conditions there is no mitogen-increase in pH\textsuperscript{ln} (2, 13, 47). Recently, Perona and Serrano (40) showed that transfection of NIH-3T3 cells with the gene for yeast H\textsuperscript{+}-ATPase renders them tumorigenic; and these cells maintain a high pH\textsuperscript{ln} under physiological conditions (14). Therefore, at least in this artificial construct, it seems clear that the presence of a H\textsuperscript{+}-ATPase in the plasma membrane is responsible not only for the high pH\textsuperscript{ln}, but also for the tumorigenicity. It should be noted that, in this system, the H\textsuperscript{+}-ATPase is electrogenic and also hyperpolarizes the plasma membrane potential (41).

The present study was undertaken to determine whether H\textsuperscript{+}-ATPases are present in the plasma membrane of naturally occurring human tumor cells. The tumor cells used in this study form colonies in soft agar (Fig 1). Furthermore, all but one of these tumor cells maintain a higher pH\textsuperscript{ln} than non-tumor cells, with the only exception being the giant cells (Fig 2). Additionally, some tumor cells maintain a
pH\textsuperscript{in} > pH\textsuperscript{ex}. Similar results of a higher pH\textsuperscript{in} than pH\textsuperscript{ex} in tumor cells have been reported elsewhere (7, 14, 26, 36, 37, 40). To our knowledge, only in few cases a high pH\textsuperscript{in} has been observed in naturally occurring tumors (21, 37). The pH\textsuperscript{in} measurements in Fig 2 were performed using the membrane-permeable dye, SNARF-1(AM) in cells attached to coverslips. We have also measured pH\textsuperscript{in} in these tumor and non-tumor cells in suspension using the dye pyranine (12, 15), and have obtained similar results, i.e. a higher pH\textsuperscript{in} in tumor than in non-tumor cells. These pH\textsuperscript{in} measurements were performed under conditions that minimize the activity of Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} and Na\textsuperscript{+}/H\textsuperscript{+} exchanger, i.e. absence of HCO\textsubscript{3}\textsuperscript{-} and serum, respectively. Since the Na\textsuperscript{+}/H\textsuperscript{+} exchange is generally inactive at pH\textsuperscript{in} values > 7.25 (for a review, see 18, 31), we consider that the maintenance of a pH\textsuperscript{in} > 7.25 in these cells must be due to the presence of another proton transporter, such as a H\textsuperscript{+}-ATPase or another Na\textsuperscript{+}/H\textsuperscript{+} exchange with a more alkaline "set point" (17, 18).

If a H\textsuperscript{+}-ATPase is involved in the maintenance of a high pH\textsuperscript{in} in naturally occurring tumor cells, we might expect that these cells would have a high metabolic rate, in analogy to our observations made in the artificial construct containing yeast H\textsuperscript{+}-ATPase (14). Indeed, the tumor cells studied here have a high rate of glycolysis when compared to non-tumor cells (Fig 3). These data are in agreement with those reported in the literature (42, 45). The only exception to this generalization
was found in the giant cell line, where the rate of lactic acid production is similar to that found in non-tumorigenic cells (c.f. Fig 3). It is worthwhile to mention that the pH<sub>in</sub> in this particular cell line is also comparable to the one observed in non-tumor cells (vide supra).

Previous studies in our lab, using non-specific H<sup>+</sup>-ATPase inhibitors have suggested that a vacuolar type H<sup>+</sup>-ATPase is responsible for the maintenance of a high pH<sub>in</sub> in some tumor cells (26). In the present study, we have screened for the presence of H<sup>+</sup>-ATPases in both tumorigenic and non-tumorigenic cells by using inhibitors with high specificity: Bafilomycin is a V-type H<sup>+</sup>-ATPase inhibitor (6, 46), and SCH 28080 is a P-type H<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor (30). The decrease in pH<sub>in</sub> observed upon bafilomycin treatment in mesothelioma and RN1a cells shown in Fig 4A and 4C, suggests that a V-type H<sup>+</sup>-ATPase is involved in maintaining the high pH<sub>in</sub> in these cells. The effect of this drug on pH<sub>in</sub> in these cells is concentration-dependent (Fig 5A). Similar results were obtained in five out of fifteen human tumor cell lines studied (Fig 5A, table 1). In contrast, the pH<sub>in</sub> of non-tumorigenic NIH, N-MUT, human foreskin fibroblasts, and normal esophageal cells are not affected by this drug (c.f. Fig 4B, Fig 4D, Fig 5A, table 1).

For the purpose of analysis, let us consider that pH changes ≤ 0.05 pH unit are insignificant, both technically and biologically. Therefore, there are only a few
instances where the pHcyt was significantly affected by these drugs. Bafilomycin affected pH regulation in only the RN1a, mesothelioma, leiomyosarcoma, lung carcinoma, breast-I cancer, and melanoma-I cells (c.f. Fig 5A, table 1). In the case of SCH 28080, only NIH and N-MUT cells were affected (c.f. Fig 5B). We interpret these data to suggest that the mouse cells generally have a SCH 28080-sensitive H⁺-ATPase at their plasma membrane. However, the presence of an additional (yeast) H⁺-ATPase in RN1a "masks" the effects of SCH 28080 in these cells. On the other hand, none of the human cell lines tested appear to contain SCH 28080-sensitive activity. Importantly, five out of fifteen human tumor cell lines (mesothelioma, leiomyosarcomna, lung carcinoma, breast-I cancer, and melanoma-I cells) are bafilomycin-sensitive, suggesting that a V-type H⁺-ATPase in their plasma membrane might be responsible for their tumorigenicity.

In mammalian cells, V-type H⁺-ATPases have been demonstrated in the membrane of several intracellular organelles, i.e. lysosomes, secretory granules, synaptosomas, and the cisternae of the Golgi apparatus (29, 33, 34). These H⁺-ATPases that normally pump H⁺ from the cytoplasm into the lumen of the organelle are also inhibitable by bafilomycin (5, 33, 34). Thus, it is possible that the cytoplasmic acidification in the bafilomycin sensitive cells could result from inhibiting the translocation of H⁺ into intracellular organelles mediated by H⁺-ATPases in vacuoles, rather than at the plasma membrane. If bafilomycin were
eliciting acidification of the cytosol by inhibiting H⁺-ATPase at the vacuole, then it should also lead to vacuolar alkalinization. We have examined this possibility by using measuring pH⁰⁺ and pH vac simultaneously and have found that bafilomycin has a variety of effects on these compartments. For example, there is no effect on either pH⁰⁺ nor pH vac in human normal foreskin fibroblasts (c.f. Fig. 9A). On the other hand, lung carcinoma cells respond to bafilomycin with a decrease in pH⁰⁺ and an increase in pH vac. There are two possible explanations for these data: bafilomycin inhibits H⁺ transport at both the plasma membrane and the vacuole; or (2) H⁺ released by inhibition of V-H⁺-ATPase at the endosome alone causes acidification of the cytosol of these cells. Unlike the lung cells, breast-II cancer cells (from a different patient than that shown in Fig 9B, table 1), exhibit a large alkalinization in the endosome/lysosome compartment with no effect at the cytoplasmic compartment. Since pH⁰⁺ is not decreased in the breast-II cells, even though the pH vac increased, it is not likely that inhibition of V-H⁺-ATPase at the endosome/lysosome is responsible by itself for acidification of pH⁰⁺ in the lung carcinoma cells. Acidification of the cytosol by release of vacuolar H⁺ also would imply a very large difference in the buffering capacity of these two compartments, given their relative volumes (Fig. 9B, and C). Analysis of the buffering capacities on these compartments is technically difficult. To date, only mesothelioma cells have shown a clear inhibition at the plasma membrane, with little or no effect at the endosome/lysosome compartments (Fig 9D, table 1). In this particular case
the observed effect would strongly argue against inhibition of a H⁺-ATPase at the endosomal/lysosomal membrane as being responsible for the pH\textsubscript{ln} decreases observed upon bafilomycin treatment. We currently hypothesize that V-type H⁺-ATPases are functionally expressed in the plasma membrane because of missorting of normal cellular constituents. This missorting could result from alterations in the cytoskeleton, a leader sequence, or in a "chaperon". Recently, Goldstein et al., (16) demonstrated that the transforming protein E5 of bovine papilloma virus selectively binds to the 16K subunit of the V-type H⁺-ATPase. It is not known whether this interaction alters the subcellular distribution of these H⁺-pumps. We are currently addressing this question.

**SUMMARY**

All but one of the tumor cells studied have a higher pH\textsubscript{ln} and a higher rate of lactic acid production than the non-tumor cells. Because these measurements were performed in the absence of HCO₃⁻ and serum, it can be argued that the high pH\textsubscript{ln} cannot arise from Na⁺/H⁺ exchange or HCO₃⁻-mediated mechanisms alone. Furthermore, our data suggest that bafilomycin selectively affects pH\textsubscript{ln} in a subset of these tumor cells. Five out of fifteen human tumor cells studied responded to bafilomycin, a vacuolar type H⁺-ATPase inhibitor, with decreases in pH\textsubscript{cpl}. We propose that this is the V-type H⁺-ATPase which is responsible for maintaining the
high pH\textsubscript{in} observed in these cells. This assertion is further supported by the simultaneous measurement of cytoplasmic and endosomal/lysosomal pH, where addition of bafilomycin to these cells resulted in decreases in pH\textsubscript{end} associated with either increases or no changes in pH\textsubscript{vac}. On the other hand, it is clear that other factors are involved in maintenance of high pH\textsubscript{cyt} in the other tumorigenic cell types. It is worthwhile to mention that our assertion of the presence of a plasmalemmal (V-type) H\textsuperscript{+}-ATPase in mesothelioma cells, relies solely on the activity of a drug whose spectrum of activities is not well characterized. "There is nothing as specific as an uncharacterized drug" (E. Racker) Therefore, more direct evidence will be required before the presence of (V-type) H\textsuperscript{+}-ATPase in the plasma membrane of these cells can be firmly established. Utilization of H\textsuperscript{+}-ATPase specific antibodies to study distinct subcellular localization may help to resolve this issue. Combined with the observations from yeast H\textsuperscript{+}-ATPase transformed cells, our results indicate that a plasma membrane V-type H\textsuperscript{+}-ATPase may play a role in the mechanisms of tumorigenesis in certain specific cell types.

ACKNOWLEDGMENTS.

The authors would like to acknowledge to David Camacho, Paul Fanta, and Kathy Brown for their technical assistance in some of the aspects of this work; to Dr. S.E. Salmon, Cancer Center for the use of their colony counter; to Dr. A.
Liebowitz for supplying the primary human tumor cells; to Dr. K. Altendorf for the generous gift of bafilomycin; to Dr. A. Barnett for the generous gift of SCH28080; to Dr. Rosario Perona for providing us with the RN1a and N-MUT cells; and to Drs. Ramon Serrano and E. Racker for their helpful discussions.
REFERENCES


1872.


APPENDIX III.

SIMULTANEOUS MEASUREMENT OF $pH^{in}$ AND $[Ca^{2+}]_{in}$ USING THE FLUORESCENCE OF SNARF-1 AND FURA-2: APPLICATIONS TO THE STUDY OF $pH^{in}$ REGULATION AND PROGESTERONE SECRETION.
Simultaneous measurement of intracellular pH and Ca\(^{2+}\) using the fluorescence of SNARF-1 and Fura-2.

March 25, 1991

Dear Sirs:

As a part of my Ph.D. dissertation entitled "The Role of Intracellular pH and Calcium in the Regulation of Cellular Functions", I would like to append an article that I have already published in Am. J. Physiol. 260 (Cell Physiol. 29): C297-C307, 1991. Therefore, I am requesting your permission to reproduce it.

Thank you for your assistance in this matter.

Sincerely,

Raúl Martínez-Zaguirán

March 30, 1992
Simultaneous measurement of intracellular pH and Ca$^{2+}$ using the fluorescence of SNARF-1 and fura-2

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Simultaneous measurement of intracellular pH and Ca$^{2+}$ using the fluorescence of SNARF-1 and fura-2. Am. J. Physiol. 260 (Cell Physiol. 29): C297–C307, 1991.—Upon cell stimulation with hormones and other mitogens, a variety of biochemical and physiological responses occur within the first few minutes. Changes in both intracellular pH (pH$_i$) and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) are prominent and play a major role in the signal transduction mechanism leading to the physiological response, i.e., secretion, neurotransmission, proliferation, or differentiation. However, it is not clear whether these ions work independently in the activation pathway leading to a particular physiological response. The fluorescence characteristics of most Ca$^{2+}$ indicators are pH sensitive, and quantitative estimates of [Ca$^{2+}$]$_i$ cannot be made without knowledge of pH$_i$. Thus it is desirable to have a technique to simultaneously monitor these two ions with relatively high time resolution. Here we have developed experimental conditions that allow us to use optimum emission conditions for a pH fluorescent indicator SNARF-1 and optimum excitation conditions for the Ca$^{2+}$ indicator fura-2. The fluorescence spectra of these compounds are sufficiently different to allow simultaneous measurement of pH and Ca$^{2+}$ both in vitro and in situ. We have observed simultaneous changes in both pH$_i$ and [Ca$^{2+}$]$_i$ in BALB/c 3T3 cells on treatment with the non-fluorescent Ca$^{2+}$ ionophore 4-bromo-A23187. This temporal relationship between pH$_i$ and Ca$^{2+}$ gives further credence to the interrelationship between these two second messengers in the expression of physiological responses.

BALB/c 3T3 cells; sodium-hydrogen exchanger; 4-bromo-A23187

Responses to a variety of hormones and mitogenic agents include turnover of inositol phospholipids (2, 33), activation of protein kinase C (25) and tyrosine kinases (34), and changes in intracellular pH (pH$_i$) (3, 24) and intracellular calcium concentration ([Ca$^{2+}$]$_i$) (4, 27). Although changes in pH$_i$ and [Ca$^{2+}$]$_i$ have been observed in many systems, it is not yet clear whether these ionic events work independently in the activation pathway leading to a particular physiological response. One of the fundamental problems in studying these ionic events is the relationship between pH$_i$ and [Ca$^{2+}$]$_i$ (2–4, 24, 27, 33, 34). It is therefore desirable to have a technique that allows concomitant monitoring of these two ions with a high time resolution.

With the exception of pH measurement by nuclear magnetic resonance spectroscopy (8), fluorescence spectroscopy is one of the least biologically disruptive techniques to measure pH$_i$ and [Ca$^{2+}$]$_i$. It also permits very fast tracking of changes in intracellular ions, even at millisecond sampling rates. There are several fluorescent indicators for measuring pH$_i$ and Ca$^{2+}$ (11, 32). Most of these are commercially available in acetoxymethyl ester (AM) forms, allowing passive loading of cells without the need for microinjection, scrape loading, glass bead loading, or electroporation. These latter manipulations are capable of disrupting the cell physiology (22). Recently, a new family of pH fluorescent indicators has been developed (32). SNARF-1 is an example; it has dual emission wavelengths, a clear isoemissive point, and a good in situ acid dissociation constant (pK$_a$), tested in the present study. Even with its documented pitfalls, the most informative Ca$^{2+}$ fluorescent probe is still fura-2 (32). Some of the problems in using this dye are incomplete hydrolysis of the AM form (28), compartmentalization into organelles (20), photobleaching (1), and exclusion from the cell by anion transport mechanisms (21). Notwithstanding these problems, with the use of appropriate controls, quantitative measurements using fura-2 can be made (32).

To simultaneously monitor pH$_i$ and [Ca$^{2+}$]$_i$, the probes should have distinct excitation or emission characteristics. A further requirement is that the probes have at least two excitation or emission wavelengths sensitive to the ion of interest and a third (isoexcitation or isoemissive) wavelength that is insensitive. An isoeexcitation or isoemissive point is greatly desirable, since it allows one to simultaneously determine efficiency of dye loading, dye leaking, and quenching effects. Ratiing the sensitive wavelengths should give unique values at any ion concentration. In addition, these probes should have apparent dissociation constants (K$_{app}$) in the physiological range, have a high quantum efficiency, and should be specific for the ion of interest.

In the present study, we have developed experimental conditions that allow us to use optimum emission conditions for a pH fluorescent indicator, SNARF-1, and optimum excitation conditions for the Ca$^{2+}$ indicator fura-2. The fluorescence spectra of these compounds are sufficiently different to allow simultaneous measurement of pH and Ca$^{2+}$ both in vitro and in vivo. We show that...
the fluorescence ratio of SNARF-1 is unaffected by Ca\textsuperscript{2+}, the presence of fura-2 in the samples, or by dye concentration. Although the fluorescence ratio of fura-2 is insensitive to the presence of SNARF-1, it is slightly affected by pH and dye concentration. We have performed in situ calibration curves to obtain the $K_{dual}$ and $pK_a$ values for fura-2 and SNARF-1, respectively. Furthermore, we have observed simultaneous increases in both [Ca\textsuperscript{2+}], and pH, in BALB/c 3T3 cells on stimulation with the nonfluorescent Ca\textsuperscript{2+} ionophore 4-bromo-A23187 (4-BrA23187), providing support to the interrelationships between these two ions in the expression of physiological responses.

**Materials and Methods**

**Fluorescence measurements.** All in vitro fluorescence measurements were performed in a temperature-controlled stirred cuvette unit housed in an SLM8000C ISLM, Urbana, IL) at 37°C using 4-nm bandpass slits and an external rhodamine standard as a reference.

**Cell culture.** BALB/c 3T3 mouse embryo fibroblasts were obtained from American Type Culture Collection, Rockville, MD (ATCC CCL 163). These cells were cultured as described by Gillies et al. (10). Briefly, the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY), supplemented with 10% Nu-serum (Collaborative Research, Bedford, MA), in a 5% CO\textsubscript{2} atmosphere at 37°C. Cells were grown in T-75 culture flasks and passed biweekly at an optimum density of 2 x 10\textsuperscript{5} cells/cm\textsuperscript{2} flask. Cells were subcultured for experiments onto glass cover slips (9 x 22 mm) in a 100-mm dish at 5 x 10\textsuperscript{5} cells/dish.

**EPO/ATAA6F12 (EPO) hybridoma cells were generously donated by Amgen (Boulder, CO). These cells were cultured in RPMI 1640 (Sigma Chemical, St. Louis, MO) as suspension cultures, supplemented with 10% Nu-serum, in a 5% CO\textsubscript{2} atmosphere at 37°C. Stocks were grown in a 250-ml spinner flask and passed biweekly at an inoculation density of 5 x 10\textsuperscript{5} cells/ml. For experiments, cells were seeded at 2 x 10\textsuperscript{4} cells/1.0-liter spinner flask. As needed, cells were centrifuged at 400 g for 5 min at 4°C. The pellet was resuspended in the appropriate buffer. Aliquots of 2 x 10\textsuperscript{3} cells/0.0 ml (fluorometer cuvette) were used for Ca\textsuperscript{2+} or pH measurements. Fluorescence measurements were performed in a temperature-controlled continuously stirred cuvette.

**Small leucine ovine cells were obtained from superovulated Western range ewes and separated by elutriation as described elsewhere (15). For experiments, cells were handled under similar conditions as described for EPO cells at a final cell density of 2 x 10\textsuperscript{5} cells/ml of the appropriate buffer.**

**Primary human leiomysarcoma cells were provided by Dr. Albert Liebowitz (UA Cancer Center). These cells were grown for experiments, essentially as described for BALB/c 3T3 cells, except that the glucose concentration in the medium was 23 mM.**

**Chemicals.** Fluoroprobes were obtained from Molecular Probes (Eugene, OR). For all fluorescence measurements, a fresh batch of dye was used. Unless otherwise stated, all other chemicals were obtained from Sigma Chemical.

**Buffers.** Cell suspension buffer (CSB) contained (in mM) 1.3 CaCl\textsubscript{2}, 1 MgSO\textsubscript{4}, 5.4 KCl, 0.44 KH\textsubscript{2}PO\textsubscript{4}, 110 NaCl, 0.35 NaH\textsubscript{2}PO\textsubscript{4}, 5 glucose, 2 glutamine, and 25 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), at a pH of 7.15 at 37°C. The 0-Ca\textsuperscript{2+}-buffer (KEGTA) contained (in mM) 110 KCl, 20 3-(N-morpholinosulfonyl)propanesulfonic acid (MOPS), 20 NaCl, and 10 mM K\textsubscript{2}H\textsubscript{2}ethanedioylbis(aminomethyl ether)-N,N,N’,N’’-tetraacetic acid (EGTA). Calcium-saturated buffer (CaeGTA) contained (in mM) 146 KCl, 10 NaCl, and 10 K\textsubscript{2}CaGTA. High K’ buffer contained (in mM) 146 KCl, 5 glucose, 2 glutamine, and 10 HEPES, 10 2-(N-morpholino)ethanesulfonic acid (MES), and 10 bicarbonate.

**Culture preparation.** Subconfluent 3T3 cells cultures grown on cover slips were used for most of these experiments. These cells were washed three times with CSB. Subsequently, two cover slips (containing cells) were incubated for 30 min at 37°C in a 5% CO\textsubscript{2} atmosphere with 3 ml of CSB containing 20 μM SNARF-1/AM and 2 μM fura-2/AM. The AM forms of these dyes are lipophilic and cell permeant. Cellular esterases cleave the ester groups of these AMs to yield the free acids, which are more impermeant and therefore “trapped” within the cells (32). After the 30-min incubation, buffer was removed by aspiration and the cells were washed three times with CSB containing 0.2% (vol/vol) Nu-serum (0.2% CSB), followed by a second incubation in 0.2% CSB for 30 min to allow for the complete hydrolysis of these dyes. After this time, two cover slips were placed back to back in a holder/perfusion device, which was subsequently inserted into the fluorometer cuvette (10).

Sample temperature was maintained at 37°C by keeping both the water jacket and the perfusion buffer at 37°C using a circulating water bath (Lauda model RM20, Brinkmann Instruments, Westbury, NY). All fluorescence measurements in cells were performed under ambient CO\textsubscript{2}.

**Ratio method.** In the ratio method, ion-sensitive excitation or emission wavelengths are selected that increase or decrease on cation binding. After correction for auto-fluorescence, the ratio of the fluorescence at these two wavelengths can be used to determine the extent of dye bound and hence the free ion concentration (7, 12) by

\[
pH = pK_a + \log(S_{CSB}/S_{CSB}) + \log([R - R_{\text{max}}]/[R_{\text{max}} - R]) 
\]

\[
[Ca^{2+}] = K_d(S_{CSB}/S_{CSB})/[R - R_{\text{max}}]/[R_{\text{max}} - R] 
\]

where $R$, $R_{\text{max}}$, and $R_{\text{min}}$ are the measured minimum and maximum ratios, respectively, and $S_{CSB}$ and $S_{CSB}$ are the fluorescence values for the free and bound forms of the dye, respectively, at the denominator wavelength. In the case of SNARF-1, R increases with deprotonation, so $R_{\text{max}}$ represents the ratio of fully deprotonated dye, whereas $R_{\text{min}}$ is the ratio for fully protonated dye. In the case of fura-2, R increases with increasing Ca\textsuperscript{2+}, so $R_{\text{max}}$ represents fully free fura-2, whereas $R_{\text{min}}$ represents the Ca\textsuperscript{2+}-fura-2 chelate.

**pH measurements.** The fluorescence of SNARF-1 was...
excited at 534 nm and monitored using emission wavelengths of 584, 600, and 644 nm. The fluorescence at 600 nm (incoherent point) is proportional to the total amount of intracellular SNARF-1. The ratio of fluorescence intensities at emissions of 644 and 584 nm was converted to pH values using Eq. 1. In vitro pH values used for calibration were obtained with a Beckman model 71 pH meter, using a Beckman gel-filled combination electrode. The electrode was calibrated at two known pH values using commercially prepared standards from VWR Scientific (San Francisco, CA).

Intracellular Ca²⁺ determinations. Fluorescence of fura-2 was excited alternately at 340, 360, and 380 nm at a constant emission wavelength of 510 nm. Fluorescence at 360 nm (isosbestic point) is proportional to the total amount of intracellular fura-2 and can be used to evaluate possible quenching. [Ca²⁺] was calculated from the ratio of intensities at excitation of 340 and 380 nm (Eq. 2). In vitro calibration curves with fura-2 were performed either by using Ca²⁺-EGTA buffers of defined compositions (13) or by varying [Ca²⁺] using Chelex (Sigma Chemical) in the presence of trace Ca, as described by Lattanzio (19). Corrections of Ca²⁺-EGTA association constants for pH, temperature, and ionic strength were performed as described by Harrison and Bers (14).

In situ calibration of SNARF-1. Calibration of intracellular SNARF-1 in the cells was performed as described previously for pyranine (10). Briefly, SNARF-1-loaded cells were perfused with high K⁺ buffer plus 6.8 µM of the K⁺-H⁻ ionophore nigericin. Under these conditions, pH of the cells was equal to the extracellular pH (pHe). By varying pH, between pH 6.0 and 8.0, one can obtain the phr of SNARF-1 from these in situ calibration curves. The K⁺ concentration in high K⁺ buffer is equal to that present in BALB/c 3T3 cells (10).

In situ calibration of fura-2. For calibration of intracellular fura-2, we used the nonfluorescent analogue of the Ca²⁺ ionophore A23187, 4-BrA23187 (Molecular Probes), to collapse the Ca²⁺ gradient (6). Fura-2-loaded 3T3 cells were perfused with Ca²⁺-EGTA buffer at various pHs with variable free Ca²⁺ concentrations ranging from 0 to 250 µM and were treated with 2 µM of 4-BrA23187. Alternately, fura-2-loaded 3T3 cells were incubated for 10 min in the presence of 2 µM 4-BrA23187 and 6.8 µM of nigericin, a high-K⁺ buffer with selected Ca²⁺ concentrations. Subsequently, cover slips containing cells were transferred to the fluorometer cuvette and perfused continuously with their respective high-K⁺ CaEGTA buffer containing 4-BrA23187 with or without nigericin. Control studies wherein 4-BrA23187 was sequentially increased in concentration showed that 2 µM was sufficient to provoke the maximum increase in fluorescence ratio. Under these conditions (e.g., in the presence of nigericin) the Ca²⁺ changes imposed by A23187 were continuous and not transient (see Fig. 8A).

Fluorometric data collection and data analysis. For simultaneous pH and Ca²⁺ measurements, data were collected in a continuous acquisition mode where the emission and excitation modes were alternated as follows: emission at 510 nm with excitation wavelengths of 340, 360, and 380 nm (fura-2 conditions) followed by excitation at 534 nm with acquisitions of the emissions at 584, 600, and 644 nm (SNARF-1 conditions). This cycle lasts 0.266 min and was repeated as many times as necessary. Data were translated to ASCII format for manipulation and analysis.

Absorbance measurements and inner filter corrections. In vitro experiments, absorbance spectra were obtained under the same experimental conditions used for fluorescence measurements, in a final volume of 3.0 ml, using a pathlength of 1 cm (Spectronic 2000, Bausch & Lomb). These values were used to correct for inner filter effects according to

\[ F_{corr} = (F_{obs} - (\text{antilog}(A_\lambda + A_m))/2) \]

where \( F_{corr} \) is the corrected fluorescence value, \( F_{obs} \) is the fluorescence obtained at the particular wavelength of interest, and \( A_\lambda \) and \( A_m \) are the absorbances at excitation and emission wavelengths, respectively (18). From absorbance data, extinction coefficients for both fura-2 and SNARF-1 were determined at a variety of wavelengths at different pHs and Ca²⁺ levels (Table 1).

Data analysis. Conversions of ratio values to both pH and Ca²⁺ were performed with Lotus 1-2-3 (Lotus Development) by using Eqs. 1 and 2 and were plotted using commercial computer software (SigmaPlot Scientific Graph System, Jandel Scientific). Analysis of both in vitro and in situ calibration curves, as well as the estimated

### Table 1. Extinction coefficients of fura-2 and SNARF-1

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<th>pH</th>
<th>Extinction coefficients: Fura-2, M⁻¹·cm⁻¹</th>
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Values are means ± SD; n = 3 experiments. Data were obtained from a set of curves similar to those shown in Fig. 4, A and B, and were calculated from absorbance values. Data were fitted by least-squares regression analysis using computer software (MINOS, Merleau-Sci- entific Software, Salt Lake City, UT). Absorbance was measured under the same experimental conditions used for fluorescence measurements, in a final volume of 3.0 ml, using a spectrophotometer (Spectronic 2000, Bausch & Lomb). Extinction coefficients for fura-2 at 510 nm were consistently < 60 M⁻¹·cm⁻¹ at all Ca²⁺ and pH values.
RATION OF THE pKs AND Rmax AND Rmin VALUES FOR SNARF-1 AND FURA-2, WERE PERFORMED USING EQS. 1 AND 2 TO FIT THE EXPERIMENTAL R VALUES AT VARIOUS pHs AND [Ca++] RESPECTIVELY, BY THE SIMPLEX METHOD AND BY LEAST-SQUARES REGRESSION ANALYSIS (MINSQ, MICRO-MATH SCIENTIFIC SOFTWARE, SALT LAKE CITY, UT).

RESULTS

Effect of pH on spectrum of SNARF-1. SNARF-1 in its free acid form was dissolved in high-K+ buffer without nigericin, pH was varied between 6.0 and 8.0, and emission spectra were recorded at various excitation wavelengths. Although SNARF-1 offers a wide variety of possible combinations of excitation or emission wavelengths, the largest dynamic range for pH measurement was obtained at an excitation wavelength of 534 nm, with emission wavelengths of 584 and 644 nm. The prominent features of these spectra are the isoemissive point at 600 nm and the peaks at 584 and 644 nm that decrease and increase, respectively, as the solution pH is increased (Fig. 1A). The in vitro spectra of SNARF-1 are not significantly different than those obtained in situ after deesterification of SNARF-1/AM (Fig. 1B).

Effect of Ca++ on spectrum of fura-2. Fura-2 in its free acid form was dissolved in Ca++-EGTA buffers at various free Ca++ concentrations at pH 7.1. The highest dynamic range for Ca++ measurement was obtained by measuring the ratio of the excitation wavelengths at 340 and 380 nm, using an emission wavelength of 510 nm (Fig. 1C). The slight shoulder at 392 nm is generally thought to be a contaminant of the free acid; however, it is sometimes observed in the in situ deesterified fura-2/AM solutions (Fig. 1D). These data are shown primarily to illustrate the nonoverlap of the SNARF-1 and fura-2 spectra and the relative effects of varying pH and Ca++, respectively. From these and similar in situ data, we estimate the intracellular concentrations to be ~0.5 mM for fura-2 and 0.1 mM for SNARF-1. These calculations were made assuming 10 nM/µg protein and 300 µg protein/sample.

Ratio measurements. The ratio of intensities at 644/584 nm of the SNARF-1 fluorescence, as well as the 340/

![Fig. 1.](image-url)
SIMULTANEOUS MEASUREMENT OF pH AND Ca$^{2+}$

**FIG. 2.** A: effect of SNARF-1 on fura-2 fluorescence ratio. Fura-2 (1 μM; free acid) was dissolved in Ca$^{2+}$-EGTA buffers of defined Ca$^{2+}$ composition (pH = 7.10) (see MATERIALS AND METHODS) in either presence or absence of 2 μM SNARF-1, and excitation spectra were recorded. B: effect of fura-2 on SNARF-1 ratio. SNARF-1 (2 μM; free acid) was dissolved in high-K$^+$ buffer at various pH values, and its emission spectra were obtained in both presence and absence of fura-2 (1 μM).

**FIG. 3.** Effect of pH on $K_{app}$ of Ca$^{2+}$ binding to fura-2. $K_{app}$ values of calibration curves were obtained by fitting data to Eq. 2. Each point represents mean ± SD of 3–11 different Ca$^{2+}$ concentrations at indicated pH value. $H^+$ and Ca$^{2+}$ association constants for EGTA were calculated as described in MATERIALS AND METHODS.

The excitation wavelength at 360 nm (isoexcitation point) for fura-2 and the emission wavelength at 600 nm (isoemissive point) for SNARF-1 can be used to monitor for efficiency of cell loading, possible quenching effects, and/or artifacts.

**Dye selectivity.** We next tested the effects of fura-2 on pH measurement by SNARF-1 and for the effects of SNARF-1 on Ca$^{2+}$ measurement by fura-2. As illustrated in Fig. 2A, SNARF-1 does not affect the fluorescence ratio of fura-2 at Ca$^{2+}$ concentrations ranging from 0 to 250 μM (Fig. 2A; data not shown). In a likewise manner, fura-2 does not affect the fluorescence ratio of SNARF-1.
SIMULTANEOUS MEASUREMENT OF pH AND Ca++

**SNARF-1** \((\lambda_{ex} 534)\)

**FURA-2** \((\lambda_{em} 510)\)

**Effect of dye concentration.** To correct for inner filter effects, extinction coefficients were determined for both fura-2 and SNARF-1 (Table 1). As shown in Table 1, the extinction coefficients of fura-2 at 340 nm tend to increase with increasing Ca++ concentration. In contrast, the extinction coefficients of fura-2 at 360 and 380 nm decreased with increasing Ca++ concentration. This phenomenon was observed at all pH values tested. In the case of SNARF-1, increases in the extinction coefficients with increasing pH were observed at 584, 600, and 644 nm. No effect of pH on the extinction coefficient at 534 nm was observed, nor were there any effects of Ca++ on the extinction coefficients at any wavelength (Table 1; data not shown).

As shown in Fig. 4A, the ratio for fura-2 at high [Ca+++] decreases with increasing dye concentration, whereas the ratio in the absence of Ca++ is relatively unaffected. This phenomenon is observed at all pH values (data not shown). As a consequence, the dynamic range for Ca++ measurements decreases with increasing dye concentration. The values in Fig. 4A are corrected for inner filter effects using data in Table 1. In contrast, SNARF-1 fluorescence ratio is not affected by dye concentration once fluorescence is corrected for inner filter (Fig. 4B).

**Simultaneous measurements in vitro.** Figure 5 shows the important features of SNARF-1 and fura-2 fluorescence as they are used in simultaneous pH and Ca++ measurements. Figure 5A illustrates that the isoemissive point \((\lambda_{em} = 600 \text{ nm})\) for SNARF-1 is not significantly affected by varying pH (Fig. 5A) in the presence of 2 \(\mu\text{M}\) fura-2, whereas the fluorescence at emission wavelengths of 584 and 644 nm decreases and increases, respectively, with increasing pH. These data can be expressed in terms of ratio, as shown in Fig. 5B. Likewise, the isoeexcitation point \((\lambda_{ex} = 360 \text{ nm})\) of fura-2 is not affected by varying Ca++ in the presence of 3 \(\mu\text{M}\) SNARF-1 at a constant pH (i.e., 7.10) (Fig. 5C), whereas fluorescence emissions at 340 and 380 nm increase and decrease, respectively, with
increasing [Ca\(^{2+}\)]. This causes an increase in the 340/380-nm ratio (Fig. 5D). The behavior of both of these dyes is therefore unaffected by the presence of the other dye in the same in vitro system.

Figure 6 summarizes our in vitro results using simultaneous measurements of pH and Ca\(^{2+}\) in a solution containing 2 μM fura-2 and 3 μM SNARF-1 in Ca\(^{2+}\)-EGTA buffer at various free Ca\(^{2+}\) concentrations at a single pH and at various pH values in high-K\(^{+}\) Ca\(^{2+}\)-free buffer. The changes in the 644/584-nm ratio observed between different Ca\(^{2+}\) concentrations are due to slight differences in pH of the different Ca\(^{2+}\)-EGTA buffers used to generate these data and do not reflect an effect of Ca\(^{2+}\) on fluorescence ratio of SNARF-1. However, the 340/380-nm fluorescence ratio for fura-2 consistently decreases as pH is increased. This decrease is consistent with the effects of pH on FURA fluorescence and absorbance (e.g., increasing inner filter).

Calibration of dyes in situ. In situ calibration of SNARF-1 in 3T3 cells was performed as described by Giulano and Gillies (10) for pyranine, using nigericin to set pH\(_m\) = pH\(_o\) (Fig. 7A). From such calibration curves we determined that pH\(_m\) = 7.34, R\(_{exc}\) = 0.437, and R\(_{emi}\) = 1.859. These values are not significantly different than those determined in vitro. One of the drawbacks of SNARF-1 when compared with other pH fluorescent dyes, such as pyranine or 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescin (BCECF), is the difficulty in obtaining reliable in situ calibration points at pH > 7.5. The reasons for this anomalous behavior of the dye in situ, but not in vitro, are not immediately apparent.

We have used 4-BrA23187, a nonfluorescent Ca\(^{2+}\) ionophore, in combination with nigericin and high K\(^{+}\) to generate physiologically relevant calibration curves for intracellularly trapped fura-2. Addition of 2 μM 4-BrA23187 to 3T3 cells continuously perfused with Ca\(^{2+}\)-EGTA buffers of various Ca\(^{2+}\) concentrations results in a rapid increase in the fluorescence of fura-2, which is proportional to the Ca\(^{2+}\) concentration being tested. A concentration of 2.0 μM 4-BrA23187 is sufficient to induce the maximum change in fura-2 fluorescence (data not shown). With the variance of external Ca\(^{2+}\) and pH in the presence of these ionophores, the \(K_{d_{e_{x_{p}}}}\) values of the fura-2-Ca\(^{2+}\) complexes were determined (Fig. 7B). Use of nigericin is essential in calibrating fura-2 in situ, since in many cells varying Ca\(^{2+}\) levels can affect the pH, which in turn also affects fura-2 fluorescence (cf. Fig. 8A). We have used similar experimental protocols for the in situ calibration of fura-2 in other cell lines and have determined similar behavior of AM dyes in cell toxicity cannot be discarded, we have evaluated their effect on
both cell viability and cell proliferation. Cell viability (as evidenced by trypan blue exclusion) was not altered with concentrations up to 100 μM SNARF-1/AM for periods of up to 4 h, which was the approximate time frame of our experiments. However, the presence of this dye for as little as the normal time required for cell loading (i.e., 30 min at 37°C) at concentrations as low as 1 μM, followed by washout of the dye, results in an arrest of the cell proliferation within the first 24 h (data not shown). The mechanisms of such cytotoxicity are not apparent but may be related to ATP depletion or to aldehyde formation on cleavage of the AM form of these dyes (31). Further studies are required to understand the molecular mechanisms that underlie the cytotoxicity of these dyes.

**Simultaneous measurements in situ.** We tested the usefulness of this system to simultaneously monitor changes in pH, and [Ca$^{2+}$]$_{in}$ in a variety of cell lines. Figure 8, A and B, shows the effect of 4-BrA23187 on pH and [Ca$^{2+}$]$_{in}$ in serum-deprived quiescent 3T3 cells.
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FIG. 9. Simultaneous evaluation of effect of varying pH on fura-2 fluorescence in primary leiomyosarcoma cells. Leiomyosarcoma cells were grown on cover slips, as described in MATERIALS AND METHODS, and loaded with 2 μM fura-2 and 2 μM SNARF-1. Subsequent handling was as described for BALB/c 3T3 cells (see Fig. 8A). Perfusion was begun with a solution containing 10 mM NHCl in ESB. At time indicated, medium was exchanged for NHCl-free CSB. Fifteen minutes thereafter, medium was exchanged for CSB containing 5 μM 4-BR-A23187.

The treatment of these cells with ionophore in bicarbonate-free CSB at a pH of 7.10 results in simultaneous and transient increases in fura-2 and SNARF-1 ratios (Fig. 8A). The [Ca]**, calculated directly from these ratios using literature values of Kd(fura) = 224 nM (13) are illustrated by the dotted line. In nominally Ca**-free medium, addition of 4-BR-A23187 induces a transient decrease in SNARF-1 ratio and a continuous increase in the ratio of fura-2 (dotted line, Fig. 8B). The solid lines in Fig. 8, A and B, represent the [Ca]**, calculated using pH-corrected Kd(fura) values (see DISCUSSION). Figure 8, C and D, shows that 4-BR-A23187 or prostaglandin F, induces permanent increases in Ca** in EPO and ovine large luteal cells, respectively, with no concomitant changes in pH. Figure 9A illustrates the effect of perturbing pH on fura-2 fluorescence. In these experiments, primary human leiomyosarcoma cells were pre-treated with 10 mM NHCl. Washout of NHCl induces a rapid acidification. This treatment also results in an increase in apparent [Ca]** levels (dotted line, Fig. 9B).

DISCUSSION

The current literature is replete with studies demonstrating that the first responses of cells to stimulation by hormones or other agents are reflected in early ionic events, involving not only changes in pH and [Ca]** but also in other physiologically important ions, i.e., K**, Na**, Mg**, and Cl- (2-4, 24, 25, 27, 33). Therefore a major effort in different laboratories has been the development of efficient techniques to measure the physiological relevance of such changes and their interrelatedness, if any, since simultaneous changes in more than one of these parameters are expected.

Attempts to simultaneously measure pH and [Ca**]** by fluorescence spectroscopy have been made by Simpson and Rink (30), using human platelets loaded with fura-2 and BCECF. We now report the simultaneous measurement of pH and Ca** by using the fluorescence of SNARF-1 and fura-2 (Fig. 1). The versatility of different excitation and emission wavelengths offered by SNARF-1 allowed us to specifically select those excitation and emission wavelengths far away from those of fura-2, minimizing the possibility of fluorescence "cross-talk." Figure 2 summarizes the results of such analysis and allows us to conclude that, under our experimental conditions, fura-2 does not interfere with the fluorescence of SNARF-1 or vice versa (Fig. 2, A and B). Furthermore, the present method offers additional advantages, since it exemplifies not only the use of ratio measurements for both pH and Ca** but also emphasizes the need to continuously monitor the fluorescence at both ion-sensitive and ion-insensitive wavelengths. These latter ion-insensitive wavelengths can be useful to check for possible artifacts, such as quenching.

Effect of dye concentration. It is commonly assumed that a fluorescence ratio corrects for variations in dye concentrations. Our data indicate that this may be an oversimplification, since self-quenching and/or inner filter phenomena may affect the fluorescence signal. Inner filter effects can be corrected and are generally not significant when working in situ, since the pathlength through cells is so small. In the case of fura-2, however, inner filter corrections do not totally account for the decrease in the fluorescence ratio of fura-2 with increasing dye concentrations (Fig. 4A). Furthermore, the effect of dye concentration on the ratio is also dependent on [Ca**]. Therefore quantitative statements regarding changes in Ca** levels require knowledge of the dye concentration, which may be gathered by fluorescence values at the ion-insensitive wavelength. In contrast, the fluorescence ratio of SNARF-1 is not affected by dye concentration (Fig. 4B).

Effect of pH on fura-2. Although most Ca** fluorophores are pH sensitive, these effects are usually confined to relatively acidic pH values (5, 13, 19, 23, 26; Fig. 3). The effect of pH on the Kd(fura) of fura-2 can be predicted by Eq. 4 with the fitting parameters discussed above. With the use of these equations and simultaneous measurement of pH, the Kd(fura) of fura-2 can be calculated at any pH and at any time and used to calculate [Ca]**. If [Ca**]** measurements are to be made under conditions where pH is >7.0, the pH-dependent corrections are minor (see Fig. 8, A-D). However, if pH falls to <7.0, pH-dependent corrections can have a significant effect on the quantitative, as well as the qualitative, data (Figs. 8B and 9).

We and others have observed the presence of fura-2-derived compounds with unusual pH sensitivity in vitro, using certain batches of fura-2 tetrapotassium salt, which contain compounds with similar spectral properties such as...
as the ester form of the dye. Because the nature of this pH-sensitive by-product is unknown, it would be most prudent to assume that they also can be formed in situ. Therefore whenever a change in fura-2 fluorescence is observed concomitant with changes in $pH_n$, the pH dependency of the dye in situ must be corrected before the fluorescence changes can be ascribed to a change in $[Ca^{2+}]_{i}$.

**Calibration in situ.** We have used 4-BrA23187 for in situ calibration of 3T3 cells (F. Fig. 7B). Because $K_{aupt}$ of fura-2 can be affected by pH and $Ca^{2+}$ levels can affect $pH_n$ in some cell types, it may be anticipated that calibration of those "A23187-responsive" cells is not a trivial matter. Indeed, we have observed that it is straightforward to calibrate cells in situ that do not increase $pH_n$ on 4-BrA23187 treatment, such as small ovine luteal cells and a hybridoma cell line (EPOFs7); (Fig. 8). In contrast, the calibration of 3T3 cells, which do respond with an increase in $pH_n$ on A23187 treatment (Fig. 8A), is extremely difficult. A solution to this problem is to set both $pH_n = pK_a$ and $[Ca^{2+}]_{i} = [Ca^{2+}_{at}]$, by using both nigericin and 4-BrA23187 to obtain an in situ $K_{aupt}$ for fura-2 at various pHs. We have used this approach to calibrate fura-2 fluorescence in situ (Fig. 7).

**Simultaneous measurements in situ.** Treatment of 3T3 cells with 4-BrA23187 results in simultaneous increases in $pH_n$ and $[Ca^{2+}]_{i}$ (Fig. 8). Increases in both $pH_n$ and $Ca^{2+}$ have been reported in the literature in different cell types, including 3T3 cells, through nonstamaneous analysis of $pH_n$ and $Ca^{2+}$ (12, 17, 29, 33). Although changes in both $pH_n$ and $[Ca^{2+}]_{i}$ have been reported in a variety of cell types (for reviews, see Refs. 2-4, 24, 27, 33), most of these studies provide only circumstantial evidence for the relatedness of such changes. Therefore simultaneous measurements of these parameters are necessary if one wants to understand the temporal relationship, if any, between $pH_n$ and $[Ca^{2+}]_{i}$ changes.

The authors thank Dr. Patricia R. Hoyer and Julie A. Wegner for providing small ovine luteal cells. We also thank our colleagues in the laboratory for reading and commenting on our manuscript before submission. We would especially like to thank anonymous peer reviewer 3, whose comments greatly strengthened this report.

This work was supported by National Institute of General Medical Sciences Grant GM-41046. Address for reprint requests: R. J. Gillys, Dept. of Biochemistry, Univ. of Arizona College of Medicine, Arizona Health Sciences Ctr., Tucson, AZ 85724.

Received 28 August 1989; accepted in final form 24 September 1990.

**REFERENCES**

30. SIMPSON, A. W., AND T. J. Bink. Elevation of pH is not an essential step in calcium mobilization in fura-2-loaded human
SIMULTANEOUS MEASUREMENT OF pH AND Ca**


SECTION 2.

Prostaglandin $F_{2\alpha}$-induced calcium transient in ovine large luteal cells: I. Alterations in cytosolic-free calcium levels and calcium flux.

(Endocrinology, 127: 3029-3037).
March 25, 1991

Williams & Wilkins
428 E. Preston St.,
Baltimore, MD 21202

Dear Sirs:

As a part of my Ph.D. dissertation entitled "The Role of Intracellular pH and Calcium in the Regulation of Cellular Functions", I would like to append an article that I have already published as a co-author in Endocrinology 127:3029-3037, 1990. Therefore, I am requesting your permission to reproduce it.

Thank you for your assistance in this matter.

Sincerely,

Raul Martinez-Zaguilán

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Prostaglandin F<sub>2α</sub>-Induced Calcium Transient in Ovine Large Luteal Cells: I. Alterations in Cytosolic-Free Calcium Levels and Calcium Flux*

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ABSTRACT. The effect of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) on cytosolic calcium homeostasis was studied in suspensions of ovine large or small luteal cells from superovulated ewes. In large cells loaded with fura-2 (AM), resting cytosolic-free calcium ([Ca<sup>2+</sup>]<sub>i</sub>) was 62 ± 6 nM (Harlow's medium, pH 7.5), and PGF<sub>2α</sub>(0.5 μM) induced a rapid transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, to 152 ± 6 nM, which then decreased to 97 ± 6 nM within 3 min and remained at this level for the remainder of the treatment period (10-20 min). PGF<sub>2α</sub> did not alter intracellular pH (pH<i>i</i>) in cells loaded with snr-1 (AM) (pH<i>i</i> indicator). The transient nature of the [Ca<sup>2+</sup>]<sub>i</sub> increase was due, at least in part, to the ability of PGF<sub>2α</sub> to stimulate (<i>P</i> < 0.05) Ca<sup>2+</sup> efflux. In small cells, resting [Ca<sup>2+</sup>]<sub>i</sub> was 57 ± 5 nM, and no change in [Ca<sup>2+</sup>]<sub>i</sub> levels or pH<i>i</i> occurred with the addition of PGF<sub>2α</sub>. PGF<sub>2α</sub> also did not affect Ca<sup>2+</sup> efflux in small cells. Calcium uptake was not significantly altered by PGF<sub>2α</sub> in large or small cells. Data from kinetic analysis of the calcium transient was best fit to a two-compartment model consisting of a rapidly effluxing compartment and a slowly effluxing compartment. The size and rate constants were 62 ± 10 nM and 3.6 ± 1 min<sup>-1</sup>, respectively, for the rapidly effluxing compartment and 140 ± 9 nM and 0.02 ± 0.002 min<sup>-1</sup>, respectively, for the slowly effluxing compartment. These results provide evidence for a direct effect of PGF<sub>2α</sub> specifically on the ovine large luteal cell that involves alterations in [Ca<sup>2+</sup>]<sub>i</sub> and calcium flux. This effect is likely to be involved in intracellular mediation of the signal for luteal regression. (Endocrinology 127: 3029-3037, 1990)

THE CORPUS LUTEUM is responsible for the production of progesterone, which is absolutely required for the maintenance of pregnancy. This endocrine gland is transiently formed on the ovary following ovulation and undergoes regression if pregnancy does not occur. Luteal regression is associated with decreased secretion of progesterone, which is absolutely required for regression and protein synthesis (14). Both cell types secrete progesterone, but differ in hormonal regulation of steroidogenesis. Small cells appear to mediate the luteotropic effects of LH in the ovine corpus luteum because they contain a significant number of receptors for LH and few for PGF<sub>2α</sub> (2, 3). In addition, LH stimulates progesterone secretion in small cells via a cAMP-dependent second-messenger pathway (14, 15). Basal secretion of progesterone is 5 to 10 times greater in large cells than in small cells on a per cell basis (3, 15, 16). It has been suggested that the basal rate of progesterone production in large cells is at or near maximal capacity (14), and that large cells provide the major amount of progesterone produced by the corpus luteum on day 10 in the ewe (17).

Ovine large luteal cells contain the greatest number of high affinity receptors for PGF<sub>2α</sub> (2, 3); therefore, the large cell is likely to mediate luteolytic effects of PGF<sub>2α</sub> in the corpus luteum. Evidence suggests that calcium-mediated pathways may be involved in this luteolytic event. Inositol-1,4,5-trisphosphate (IP<sub>3</sub>) and protein kinase C (PKC) are involved in two branches of a calcium-mediated second-messenger pathway found in many tis-
sues (18). PGF\textsubscript{20} has been shown to stimulate the production of IP\textsubscript{3} in bovine (6, 19) and ovine (8) luteal tissue. PGF\textsubscript{20} also stimulates the translocation of PKC to the plasma membrane in rat (9) and swine (10) luteal cells. Activation of the PKC pathway by phorbol esters mimics a putative luteolytic effect of PGF\textsubscript{20}, in rat (20) and ovine (21, 22) luteal cells. Furthermore, endogenous protein substrates for calcium-dependent protein kinases were demonstrated in soluble fractions of ovine large luteal cells (23). Several recent studies have demonstrated that PGF\textsubscript{20} initiates a transient increase in cytosolic-free calcium ([Ca\textsuperscript{2+}]) followed by a sustained elevation of [Ca\textsuperscript{2+}], in suspensions of rat (24) and bovine large (25) luteal cells. A PGF\textsubscript{20}-induced increase in [Ca\textsuperscript{2+}], was also observed by computer-assisted microscopic imaging of fura-2 fluorescence in single ovine large luteal cells collected from cyclic ewes (26).

The fura-2 spectrofluorescence technique permits continuous measurement of changes in cytosolic-free calcium levels in cell suspensions, but does not provide information about alterations in transmembrane calcium flux or exchangeable calcium. PGF\textsubscript{20} increased calcium efflux in cultured swine granulosa cells (27). However, the effect of PGF\textsubscript{20} on calcium uptake or efflux has not been studied in isolated luteal cells. A comparison of alterations in calcium fluxes with changes in [Ca\textsuperscript{2+}] could provide more detailed information about calcium compartments and transport mechanisms involved in a PGF\textsubscript{20}-mediated mechanism. Therefore, the objectives of the present study are 1) to describe the effect of PGF\textsubscript{20} on [Ca\textsuperscript{2+}], in suspensions of large and small luteal cells from superovulated ewes, 2) to determine whether Ca\textsuperscript{2+}-fluxes are affected by PGF\textsubscript{20}, in these cells, and 3) to describe a compartmental model of calcium distribution in large luteal cells based on kinetic analysis of PGF\textsubscript{20}-induced [Ca\textsuperscript{2+}], response and calcium flux data.

Materials and Methods

Animals

Western Range ewes were synchronized with injections of PGF\textsubscript{20} (im, 10 mg, Lutealyse; Upjohn Company, Kalamazoo, MI) on days 12 and 3. Follicular development was induced with a subcutaneous injection of PMS (1000 IU, sc, day 2) followed on day 9 with an injection of human CG (hCG) (750 IU, iv, Sigma Chemical Co., St. Louis, MO). The experiments to be performed require large numbers of separated fractions of small and large luteal cells. Therefore, this treatment protocol was used to induce multiple ovulations so that sufficient numbers of cells could be recovered (29).

Tissue collection

 Corpora lutea were surgically collected on day 10 from superovulated Western Range ewes as described by Hild-Petito et al. (30). Corpora lutea were pooled and placed in sterile medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with (grams per liter): 0.35 NaHCO\textsubscript{3}, 4.7 HEPES, 1.0 BSA, 0.079 penicillin G, 0.1 streptomycin sulfate, 0.05 neomycin sulfate, pH 7.35, for transport to the laboratory.

Tissue preparation

Corpora lutea were decapsulated, sliced, and enzymatically dissociated into single-cell suspensions (0.4% collagenase (Worthington Biochemical, Malvern, PA); 0.0025% DNase (Sigma); complete Hanks' solution, pH 7.35 (22). Dissociation proceeded by incubation, 3 to 5 h, in a shaking water bath at 37°C until visible clumps disappeared. Corpora lutea were separated into large and small cell fractions by elutriation as previously described (28). Typically, the small cell fraction (12-22 μm) was free of large cells, and the large cell fraction (>22 μm) contained 10 to 20% small cells by number.

Intracellular calcium measurements

Aliquots of large (5 × 10\textsuperscript{3}/0.3 ml) or small (1 × 10\textsuperscript{3}/0.3 ml) cells were washed once with Hanks' medium (0.1% BSA, pH 7.15, 37°C) and incubated (30 min at 37°C in 5% CO\textsubscript{2} atmosphere) in 3-ml Hanks' medium containing 2 μM fura-2 (AM). In experiments involving simultaneous [Ca\textsuperscript{2+}], and pH measurements, cells were preloaded with both 2 μM fura-2 (AM) and 20 μM snarf-1 (AM). In experiments involving chelation of Ca\textsuperscript{2+}, cells were preloaded with both 2 μM fura-2 (AM) and 5 or 20 μM BAFTA-AM [5,6'-dimethyl bis-(o-aminophenoxyethyl)N,N,N',N'-tetraacetic acid]. At the end of dye loading, cells were washed, resuspended (6 ml Hanks' solution), and reincubated (30 min, 37°C) to allow complete hydrolysis of fura-2 (AM). After the second incubation, cells were washed, resuspended (3 ml Hanks' solution), and transferred to a temperaturecontrolled stirring cuvette unit housed in an SLM8000C spectrophotometer (SLM, Urbana, IL). All experiments were performed at 37°C (29).

Fluorescence measurements were collected using a continuous acquisition mode (emission at 510 nm, excitation at 480, 360, and 380 nm; each cycle lasted 0.064 min. The data-acquisition cycle during simultaneous measurement of [Ca\textsuperscript{2+}], and pH, lasted 0.056 min [emission at 510 nm, excitation at 340, 360, and 380 nm (fura-2 conditions), followed by excitation at 534 nm and emission at 504, 600, and 644 nm (snarf-1 conditions). The 360 and 600 nm wavelengths represent the isoexcitation and isoemission points for fura-2 and snarf-1, respectively. The fluorescence at the isoexcitation or isoemission point is proportional to the total amount of intracellular dye, and can be used to evaluate possible quenching or other artifacts. Steady state conditions were measured for 10 min before the addition of PGF\textsubscript{20} (0.5 μM, suspended in dimethyl sulfoxide) followed by measurements for an additional 10 min. At the end of the measurement period, 1 μM 4Br-A23187 (nonfluorescent analog of the Ca\textsuperscript{2+} ionophore A23187; Molecular Probes, Eugene, OR) was added to determine the maximum response of the system (data not shown). Data were translated to ASCII format and converted to [Ca\textsuperscript{2+}], using Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA). Cytosolic-free
calcium levels were calculated using the method described by Grynkiewicz et al. (30), and corrected for pH effects on fura-2 dissociation constant (K_d) (29) using:

$$[Ca^{2+}] = K_d \cdot \frac{(R - R_{free}}{(R_{max} - R)}$$

where $K_d$ is the apparent dissociation constant of fura-2 for $Ca^{2+}$ (140.9 nM); $R_{max}$ is determined when fura-2 is completely quenched (0.4783), and represents the minimum fluorescence 340/380 nm ratio; $R_{free}$ is the 340/380-nm ratio when fura-2 is saturated with $Ca^{2+}$ (0.079); and $R$ is the measured fluorescence 340/380-nm ratio. The pH-corrected values for $K_d$, $R_{max}$, and $R_{free}$ were determined by in situ calibration of small luteal cells.

One difficulty in using cell suspensions is the leakage of calcium levels due to dye leakage was subtracted from the actual [Ca^2+], values, and the corrected [Ca^2+] values were reported. Changes in intracellular pH were measured by the ratio of fluorescence intensities at emissions 644 and 584 nm (29).

Calcium fluxes

"Ca"_2 efflux. Large (2 x 10^6 cells/0.5 ml) or small (1 x 10^6 cells/0.5 ml) cells were preloaded to isotopic equilibrium with 5 nCi/ml "Ca"_2 (17.2 mCi/mg; New England Nuclear, Boston, MA) in Hanks' medium (60 min, 37 C, pH 7.15) with continuous shaking (New Brunswick metabolic shaker). At the end of incubation, media were collected by centrifugation (900 x g, 4 min, 1°C), and the progesterone content of the media was determined by RIA (28). Cells pellets were incubated at 100°C with continuous shaking (New Brunswick metabolic shaker). At the end of incubation, media were collected by centrifugation (900 x g, 4 min, 23°C), and the progesterone content of the media was determined by RIA (28). Cells pellets were immediately dispersed in medium 199, and cellular viabilities were assessed by trypan blue dye exclusion.

Statistical analysis

Corpora lutea from one to three animals were pooled in each tissue preparation. Each experiment (n) represents a different tissue preparation. Data are expressed as mean ± SE. Statistical significance between multiple treatments was determined by analysis of variance, followed, where appropriate, by Student-Newman-Keuls multiple-range testing. Differences between two means were determined by Student's t test for paired data. Significance was assigned at the 0.05 level.

Data from the recovery phase of the PGF_{2alpha}-induced [Ca^2+] transient and "Ca"_2 efflux curves were fit to compartmental models using nonlinear regression techniques. The number, size, and rate constants of the defined compartments were estimated using the Simplex method (MINSQ, Micromath Scientific Software, Salt Lake City, UT).

Chemicals

All chemicals were purchased from Sigma Chemical. Fluoroprobes were obtained from Molecular Probes. [3H]Progestrone and "Ca"_2 were purchased from New England Nuclear. The antibody to progesterone was CDN-337, which was kindly provided by Dr. G. D. Niswender.

Results

The $K_d$ of fura-2 is sensitive to pH in the physiological range (29). Therefore, an in situ calibration of fura-2 was performed using suspensions of small luteal cells (Fig. 1). The nonfluorescent analog of the calcium ionophore A23187, 4Br-A23187 (Molecular Probes), was used to collapse the Ca^2+ gradient (54). Fura-2 loaded cells were resuspended in Ca^2+-EGTA buffer, pH 7.15, treated with 1 μM 4Br-A23187, and variable free Ca^2+ concentrations (0-3 μM). Estimates of $K_d$, $R_{max}$, and $R_{free}$ values were determined using the Simplex method (MINSQ). These values were used to calculate [Ca^2+], for both large and small luteal cells.

The effect of PGF_{2alpha} on [Ca^2+], was measured in large and small cells using the Ca^2+ indicator, fura-2 (Fig. 2). Steady state [Ca^2+], was similar in large and small cells suspended in Hanks' solution (62 ± 5 nM, n = 5, and 61 ± 5, n = 3, respectively). PGF_{2alpha} (0.5 μM) initiated a transient increase in [Ca^2+], in large cells. Cytosolic-free calcium levels were initially increased (P < 0.05) to 152 ± 6 nM (n = 5) within 23 ± 2 s (n = 5) after addition of PGE_{2alpha} to large cells. Calcium levels returned to a higher
During the course of our experiments, it was noticed that both untreated large and small cells consistently exhibited a gradual increase in the 340/380-nm ratio with time. To determine if the sloping baseline observed in Fig. 2 was a gradual increase in [Ca\(^{2+}\)]\(_i\), or due to leakage of fura-2 from the cells, probenecid (2.5 mM), an anion transport inhibitor, was added to the cells at the beginning of the measurement period (Fig. 3). The slope was eliminated in the presence of probenecid.

To establish that the increase in fluorescence observed with PGF\(_{2\alpha}\) treatment was due to a true increase in [Ca\(^{2+}\)]\(_i\), several control experiments were performed. Incubation of large cells with 5 or 20 \(\mu\)M BAPTA (intracellular calcium chelator) decreased \(P < 0.05\) basal [Ca\(^{2+}\)]\(_i\), to 43 ± 6 nM (n = 2) and eliminated the PGF\(_{2\alpha}\)-induced calcium transient (Fig. 4). Because the \(K_{\text{d}}\) of fura-2 is sensitive to pH, an apparent change in [Ca\(^{2+}\)]\(_i\), might be the result of a change in intracellular pH (pHi) rather than a real change in [Ca\(^{2+}\)]\(_i\). Therefore, pHi and [Ca\(^{2+}\)]\(_i\), were simultaneously measured using the fluorescent dyes snarf-1 and fura-2, respectively. Under these conditions, it was observed that PGF\(_{2\alpha}\) induced a transient increase in [Ca\(^{2+}\)]\(_i\), with no effect on pH, in large cells (Fig. 5). No effect of PGF\(_{2\alpha}\) on [Ca\(^{2+}\)]\(_i\), or pHi, was observed in small cells (data not shown).

The effect of LH (100 ng/ml) on [Ca\(^{2+}\)]\(_i\), was also monitored in large and small luteal cells. No change in [Ca\(^{2+}\)]\(_i\), was observed with LH in either large or small cells (data not shown).
The radioactive tracer \(^{45}\text{Ca}^{2+}\) was used to monitor calcium efflux and uptake in response to PGF\(_{2\alpha}\) treatment in large and small cells. Calcium efflux was stimulated \((P < 0.05)\) at 3, 6, and 9 min in large cells incubated with 0.5 \(\mu\text{M}\) PGF\(_{2\alpha}\) (Fig. 6A). In contrast, there was no effect of PGF\(_{2\alpha}\) on calcium efflux in small luteal cells (Fig. 6B). PGF\(_{2\alpha}\) decreased \((P < 0.05)\) calcium uptake at the 15-min time point in large cells (Fig. 7A), but did not affect calcium uptake in small cells (Fig. 7B).

Kinetic analysis of the recovery phase of the PGF\(_{2\alpha}\)-induced calcium transient, monitored by fura-2 fluorescence, and \(^{45}\text{Ca}^{2+}\) efflux data in large cells can provide information about potential calcium compartments that contribute to alterations in \([\text{Ca}^{2+}]_{i}\), in response to PGF\(_{2\alpha}\). The kinetic parameters of the PGF\(_{2\alpha}\)-induced calcium transient and the calcium efflux curve are presented in Table 1. The recovery phase of the PGF\(_{2\alpha}\)-induced \([\text{Ca}^{2+}]_{i}\), transient and calcium efflux data was best described by a two-compartment model:

\[
y = A \exp\left(-k_1t\right) + B \exp\left(-k_2t\right) \tag{II}
\]

where \(A\) and \(B\) = size of the fast and slow compartment (nm), respectively, and \(k_1\) and \(k_2\) = rate constant \((\text{min}^{-1})\) for the fast and slow compartment, respectively.

The effect of PGF\(_{2\alpha}\) on secretion of progesterone was measured in large and small luteal cell suspensions (Fig. 8). Basal secretion of progesterone was similar at pH 7.35 and 7.15 in both cell types (data not shown). At pH 7.35, secretion of progesterone was not affected by PGF\(_{2\alpha}\) (0.5 \(\mu\text{M}\); 92 ± 3% control, \(n = 6\)). However, PGF\(_{2\alpha}\) significantly decreased progesterone secretion (83 ± 4% control, \(n = 6\)) in incubations of large cells when the pH of the medium was reduced to 7.15. Secretion of progesterone was not affected by PGF\(_{2\alpha}\) in small cells at either pH. Cell viability was not affected in either cell type by addition of PGF\(_{2\alpha}\) (data not shown).

**Discussion**

A PGF\(_{2\alpha}\)-induced transient increase in \([\text{Ca}^{2+}]_{i}\), has been demonstrated in mixed suspensions of rat (24) and suspended bovine large (25) luteal cells. Wiltbank et al. (26) demonstrated a PGF\(_{2\alpha}\)-induced increase in \([\text{Ca}^{2+}]_{i}\), in ovine large luteal cells from cyclic ewes using imaging of single cells. Although a transient increase in \([\text{Ca}^{2+}]_{i}\), was demonstrated, the limited time frame of sample recording in the later study may have reduced the ability to visualize the full extent of the transient increase in \([\text{Ca}^{2+}]_{i}\).

The objective of the present study was to observe the effects of PGF\(_{2\alpha}\) on redistribution (cytosolic increases and fluxes) of calcium in suspensions of ovine large or small luteal cells from superovulated ewes. A second objective was to demonstrate the existence of cellular compartments that might contribute to these redistributions. In large cells, PGF\(_{2\alpha}\) (0.5 \(\mu\text{M}\)) induced an increase in \([\text{Ca}^{2+}]_{i}\), that peaked rapidly and then declined...
respectively. The values were calculated by fitting the data from the recovery phase of the calcium transient, but not the sustained increase in calcium-mediated pathways in ovine large luteal cells from control to a sustained elevated level. Decreasing the concentration of PGF₂α to 0.05 μM reduced the height of the calcium transient, but not the sustained increase in steady state [Ca²⁺]. These results support the hypothesis that PGF₂α produces a direct cellular effect via stimulation of calcium-mediated pathways in ovine large luteal cells.

Leakage of fura-2 from loaded cells to the extracellular medium could be misinterpreted as a steady increase in [Ca²⁺], (31, 32). Fura-2 dye leakage has been reported in a variety of cell types including mouse macrophages (32) and rat peritoneal mast cells (35). Leakage of fura-2 varies with cell type and occurs in intact, viable cells (31, 36). Efflux of the membrane impermeant form of fura-2 occurs via an organic anion transporter because the leakage can be inhibited by probenecid, an organic anion transport inhibitor (32, 37). In the present study, a steady increase in the fura-2 340/380-nm fluorescence ratio was observed in both large and small cells in all experiments. Probenecid (2.5 mM) abolished the steady increase in fluorescence; therefore, the steady rise in fura-2 fluorescence was attributed to dye leakage. All reported values of [Ca²⁺], have been corrected for the increase in [Ca²⁺], due to dye leakage.

Large luteal cells were preincubated with the intracellular calcium chelator, BAPTA, to further provide evidence that the increase in fluorescence with PGF₂α was due to a true increase in [Ca²⁺]. Steady state [Ca²⁺], and the PGF₂α-induced calcium transient were reported to be blunted by incubation with 10 μM BAPTA in rat luteal cells (24). In the present experiments, incubation with BAPTA reduced steady state [Ca²⁺], and inhibited the PGF₂α-induced calcium transient in a dose-dependent manner. These results further support the hypothesis that PGF₂α triggers an increase in [Ca²⁺], in large luteal cells.

Changes in transmembrane calcium flux might contribute to alterations in [Ca²⁺], or might result from release of Ca²⁺ from intracellular compartments. To examine the effect of PGF₂α on calcium flux, steady state ⁴⁴Ca⁺ efflux and uptake studies were performed. PGF₂α stimulated ⁴⁴Ca⁺ efflux in large luteal cells at the 3, 6, and 9 min time points. As indicated above, [Ca²⁺], levels were also elevated during this period. Whether the stimulation of calcium efflux is the result of a direct effect of PGF₂α on plasma membrane calcium transport mechanisms or due to increased [Ca²⁺], cannot be determined from these experiments. No previous studies have re-
ported the effect of PGF$_2\alpha$, on calcium efflux in isolated luteal cells. In intact rat corpus luteum, PGF$_2\alpha$, did not alter calcium efflux (38). In cultured swine granulosa cells, PGF$_2\alpha$, increased the size of both a rapidly exchanging and a slowly exchanging calcium compartment without altering the rate constants of these compartments (27). The increase in compartment size was similar to the effect demonstrated by inhibitors of calcium-magnesium-dependent ATPase; thus, the author (27) suggested that an initial action of PGF$_2\alpha$, may be to inhibit calcium-magnesium-dependent ATPase.

Although PGF$_2\alpha$, increased [Ca$^{2+}$], and stimulated calcium influx in large cells, calcium uptake was not increased. Uptake of $^{45}$Ca$^{2+}$ is dependent on the rate of exchange and size of the cellular pools with which $^{45}$Ca$^{2+}$ exchanges (38). Because $^{45}$Ca$^{2+}$ uptake is dependent on more than just calcium influx, a lack of an observed increase in $^{45}$Ca$^{2+}$ uptake does not necessarily suggest that calcium influx was not altered. An increase in calcium influx could be masked by an increase in calcium efflux observed during this same time period. Alternatively, a brief initial period of stimulated calcium influx would not be detectable by the method used in these experiments. Calcium from intracellular stores, rather than extracellular calcium, appears to be involved in the PGF$_2\alpha$,-induced calcium transient in these cells (Wegner, S. A., R. Martinez-Zaguilan, R. J. Gillies, and P. B. Hoyer, unpublished results). Thus, if the contribution of extracellular calcium to the PGF$_2\alpha$,-induced calcium transient is small or nonexistent, an increase in calcium influx or $^{45}$Ca$^{2+}$ uptake would not be expected. The posttransient steady state elevation of [Ca$^{2+}$], appears to be dependent on extracellular calcium. (Wegner, J. A., R. Martinez-Zaguilan, R. J. Gillies, and P. B. Hoyer, unpublished results). Steady state [Ca$^{2+}$], levels are attained when calcium influx equals efflux; therefore, a stimulation of calcium influx could not be detectable during the new steady state phase of the PGF$_2\alpha$,-induced response.

Cellular content of $^{45}$Ca$^{2+}$ was decreased in the presence of PGF$_2\alpha$, in large cells at 15 min, a time during which [Ca$^{2+}$]$_i$, levels were elevated but at steady state. Steady state [Ca$^{2+}$], levels result when calcium influx and efflux are equal; however, alterations in $^{45}$Ca$^{2+}$ uptake could result from a change in the calcium gradient across the membrane, a redistribution of intracellular calcium, or alterations in the size of exchangeable calcium pools. More detailed calcium flux studies are required to determine the specific nature of the effect of PGF$_2\alpha$, on calcium compartments in large cells.

Nonlinear curve-fitting analyses of the recovery phase of the PGF$_2\alpha$,-induced calcium transient and calcium efflux data were performed to describe a model of the PGF$_2\alpha$,-sensitive calcium pools in ovine large luteal cells. If one assumes that recovery after an increase in [Ca$^{2+}$], is due to redistribution of Ca$^{2+}$ to multiple compartments (i.e. extracellular space, endoplasmic reticulum, calciosome, and mitochondria), then the recovery phase would be analogous to ion redistribution into cellular compartments by one or more calcium transport mechanisms and could be analyzed using the following equation:

$$y = A \exp[-(k_1)(t)] + B \exp[-(k_2)(t)] + ... + N \exp[-(k_N)(t)]$$

Data from the recovery phase of the PGF$_2\alpha$,-induced [Ca$^{2+}$] transient in large cells best fit a two-compartment model consisting of a rapidly exchanging compartment and a slowly exchanging compartment, which could include a one-compartment model with two calcium transport mechanisms: a slow and a fast calcium transporter (Eq II and Table 1). The distinction between two compartments or two calcium transporters cannot be made from the results of this study. Calcium efflux data were also best described by a two-compartment/two-calcium transporter model. However, the size of the calcium compartments described from the fura-2 ([Ca$^{2+}$]) and $^{45}$Ca$^{2+}$ flux data was different. These differences could be attributed to technical differences in the calcium compartments detectable by fura-2 (cytosolic-free calcium) as opposed to those detectable by $^{45}$Ca$^{2+}$ (exchangeable calcium). Nonetheless, the results of these kinetic analyses suggest that PGF$_2\alpha$, interacts with at least two calcium compartments in large cells. The specific identities of these compartments are currently being investigated.

Hormonal regulation can involve alterations in pH$_i$, which in turn can affect metabolic pathways and [Ca$^{2+}$], (40, 41). A Na$^\text{+}$-H$^\text{+}$ exchanger has been postulated to regulate intracellular pH$\text{+}$. In addition, the activity of the Na$^\text{+}$-H$^\text{+}$ exchanger may affect [Ca$^{2+}$], levels by increasing or decreasing the activity of the Na$^\text{+}$-Ca$^{2+}$ exchanger.
progesterone secretion at a lower external pH (pH 7.15), calcium efflux and an inhibition of calcium uptake. Whereas it did not produce a state increase in cells likely results from differences between the two changes in intercellular calcium. Progesterone secretion by these hormones in ovine small luteal cells interacts with ovine large, but not small luteal cells, to increase secretion by these hormones in ovine small luteal cells.

The variability in the response of PGF\_2\textalpha{} in ovine small luteal cells, in which LH initiated constriction during luteolysis, an event widely proposed and, hence, the luteolytic effect of PGF\_2\textalpha{} might be related to the lack of any type of response may be related to the role of changes in extracellular pH in the variability. Thus, alterations in [Ca\textsuperscript{2+}] in secretion of progesterone in large cells incubated at pH 7.15. PGF\_2\textalpha{} has been shown to bind optimally to receptors at pH 6.3 (2). Thus, PGF\_2\textalpha{}-receptor binding and, hence, the luteolytic effect of PGF\_2\textalpha{} on progesterone secretion may be enhanced by an acidic environment. Such a condition could result from PGF\_2\textalpha{}-induced vasoconstriction during luteolysis, an event widely proposed as part of its initial mechanism of action. These results provide evidence for a direct effect of PGF\_2\textalpha{} specifically on the ovine large luteal cell that involves alterations in concentration and flux of cytosolic-free calcium. At least two PGF\_2\textalpha{}-sensitive calcium pools contribute to these alterations, and the effect is likely to be involved in intracellular transduction of the signal for luteal regression.

Future studies will more rigorously investigate the effect of extracellular pH on PGF\_2\textalpha{}-induced inhibition of secretion of progesterone in large cells to determine the role of changes in extracellular pH in the physiological regulation of luteolysis.

Acknowledgments

The authors thank Ms. S. L. Marion, Mr. W. Engleberg, and Ms. G. M. Martinez for technical assistance.

References

1. McCracken JA, Glew ME, Scaramuzzi RJ 1979 Corpus luteum regression induced by prostaglandin F\_2\textalpha{}. J Clin Endocrinol Metab 59:444-446
2. Baleapu AK, Caicedo IC, Kawada K, Watt DS, Rezvani CE, Fitz TA 1989 Multiple classes of PGF\_2\textalpha{}-binding sites in subpopulations of ovine luteal cells. Biol Reprod 41:385-392
5. Waterman RA 1986 Changes in lipid contents and fatty acid compositions in ovine corpora lutea during the estrous cycle and early pregnancy. Biol Reprod 38:605-615
15. Hoyer PB, Fitz TA, Niswender GD 1984 Hormone-independent activation of adenylate cyclase in large steroidogenic ovine luteal cells does not result in increased progesterone secretion. Endocrinology 114:604-606
44. Rodgers RJ, O'Shea JD, Findlay JK 1986 Do small and large luteal cells of the sheep interact in the production of progesterone? J Reprod Fertil 75:85-94
47. Pita TA, Mock EJ, Mayan ME, Niswender GD 1984 Interactions of prostaglandins with subpopulations of ovine luteal cells II. Inhibitory effects of PGE₂ and protection by PGE₁, Prostaglandins 28:127-135
SECTION 3.

PGF$_{2\alpha}$-induced calcium transient in ovine large luteal cells: II Modulation of the transient and resting cytosolic free calcium alters progesterone secretion.

(Endocrinology, 128: 929-936).
March 25, 1991

Williams & Wilkins
428 E. Preston St.,
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Dear Sirs:

As a part of my Ph.D. dissertation entitled "The Role of Intracellular pH and Calcium in the Regulation of Cellular Functions", I would like to append an article that I have already published as a co-author in Endocrinology 128:929-936, 1991. Therefore, I am requesting your permission to reproduce it.

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Prostaglandin \( \text{F}_2\alpha \)-Induced Calcium Transient in Ovine Large Luteal Cells: II. Modulation of the Transient and Resting Cytosolic Free Calcium Alters Progesterone Secretion*

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ABSTRACT. A previous study demonstrated that prostaglandin \( \text{F}_2\alpha \) (PGF\(_\alpha\)) stimulates a transient increase in cytosolic free \( \text{Ca}^{2+} \) levels (IC\(_a\) = 1) in ovine large luteal cells. In the present study, the magnitude of the PGF\(_\alpha\) (0.5 \( \mu \text{M} \))-induced calcium transient in Hanks' medium (67 \( \pm \) 2 nm increase above resting levels) was reduced (\( P < 0.05 \)) but not completely eliminated in fura-2 loaded large luteal cells incubated in \( \text{Ca}^{2+} \)-free or phosphate- and carbonate-free medium (10 \( \pm \) 1 nm, 32 \( \pm \) 6 nm, above resting levels, respectively). Preincubation for 2 min with 1 mM LaCl\(_3\) (calcium antagonist) eliminated the PGF\(_\alpha\)-induced calcium transient. The inhibitory effect of PGF\(_\alpha\) on secretion of progesterone was reduced in \( \text{Ca}^{2+} \)-free medium or medium plus LaCl\(_3\). Resting \( \text{Ca}^{2+} \) levels and basal secretion of progesterone were both reduced (\( P < 0.05 \)) in large cells incubated in \( \text{Ca}^{2+} \)-free medium (77 \( \pm \) 4 nm; 70 \( \pm \) 6% control, respectively) or with 5 \( \mu \text{M} \) 5,5'-dimethyl bis-(O-aminophenoyl)ethane-N,N,N',N'-tetracetate acid (40 \( \pm \) 2 nm; 49 \( \pm \) 1% control; respectively). In addition, secretion of progesterone was inhibited (\( P < 0.05 \)) by conditions that increased (\( P < 0.05 \)) (\( \text{Ca}^{2+} \)) to that of LaCl\(_3\) (IC\(_a\) = 1), 120 \( \pm \) 17 nm; progesterone, 82 \( \pm \) 8% control and PGF\(_\alpha\) (IC\(_a\) = 1), 102 \( \pm \) 10 nm; progesterone, 82 \( \pm \) 3% control). In small luteal cells, resting \( \text{Ca}^{2+} \) levels and secretion of progesterone were reduced by incubation in \( \text{Ca}^{2+} \)-free Hanks (IC\(_a\) = 1), 28 \( \pm \) 2 nm, progesterone, 71 \( \pm \) 6% control, however, neither LaCl\(_3\) nor PGF\(_\alpha\) increased (IC\(_a\) = 1) levels or inhibited secretion of progesterone. The findings presented here provide evidence that extracellular as well as intracellular calcium contribute to the PGF\(_\alpha\)-induced \( \text{Ca}^{2+} \) transient in large cells. Furthermore, whereas an adequate level of \( \text{Ca}^{2+} \) is required to support progesterone production in both small and large cells, optimal progesterone production in large cells depends upon an appropriate window of \( \text{Ca}^{2+} \) (126: 929-936, 1991).

Received August 6, 1990.

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*This work was supported in part by grants HD-20413, BRSG RR-05705 (to P.B.H.), GM-40337 (to R.J.G.), HL-07249 (to J.A.W.), and USDA 86CRC-1-21223.
increase in [Ca\(^{2+}\)]\(\text{free}\), initiated by PGE\(_2\), in ovine large luteal cells might be one mechanism responsible for decreased secretion of progesterone and cell degeneration associated with luteolysis. 

The present study was conducted to further investigate the characteristics of the PGE\(_2\)-induced calcium transient with respect to possible calcium stores that may contribute to the transient. A second objective of the study was to determine if a relationship exists between the PGE\(_2\)-induced calcium transient and inhibition of secretion of progesterone in day-10 ovine large luteal cells. Finally, the relationship between steady state [Ca\(^{2+}\)]\(\text{free}\), and basal progesterone secretion was examined in both large and small luteal cells.

Materials and Methods

Animals

Western range ewes were superovulated according to our previously reported method (14).

Tissue collection

Day-10 corpora lutea were surgically collected, pooled, and placed in sterile Medium 199 (GIBCO Laboratories, Grand Island, NY) supplemented with (g/liter): 0.35 NaHCO\(_3\), 4.7 HEPES, 1.0% BSA, 0.079 penicillin G, 0.1 streptomycin sulfate, and 0.05 neomycin sulfate, pH 7.35 for transport to the laboratory.

Tissue preparation

Corpora lutea were decapsulated, sliced, enzymatically dissociated into single cell suspensions (0.4% collagenase: Worthington Biochemical, Malvern, PA; 0.0025% DNase: Sigma; complete Hanks; pH 7.35) and separated into large and small cell fractions by elutriation as reported by Hild-Peitoto et al. (14). The small cell fraction (12-22 \(\mu\)m) was free of large cells and greater than 90% viable as assessed by trypan blue dye exclusion. The large cell fraction (>22 \(\mu\)m) contained 10-20% small cells (by number) and demonstrated greater than 75% viability after elutriation.

Intracellular calcium measurements

Aliquots of large cells (5 x 10\(^6\)/ml) were washed once with complete Hanks' medium (0.1% BSA, 10 mM HEPES, pH 7.15, 37 \(^\circ\)C) and incubated for 30 min at 37 \(^\circ\)C in 5% CO\(_2\) atmosphere with 3 ml Hanks' Medium containing 2 \(\mu\)M fura-2 (AM). When LaCl\(_3\) was used, a modified medium was used in which NaH\(_2\)PO\(_4\), NaHCO\(_3\), and MgSO\(_4\) were omitted (modified Hanks' medium, MH medium, 10 mM HEPES, pH 7.15, 37 \(^\circ\)C) to prevent formation of a precipitate. At the end of the initial incubation period the cells were washed, resuspended, and reincubated (30 min) to allow complete hydrolysis of fura-2 (AM). After the second incubation the cells were washed and resuspended in 3 ml appropriate medium. The cell suspension was transferred to a temperature-controlled stirring cuvette unit housed in an SLM8000C spectrofluorometer (SLM, Urbana, IL). All experiments were performed at 37 \(^\circ\)C.

Fluorescence measurements were collected using a continuous acquisition mode where emission at 510 nm was alternated with excitation at 340, 360, and 380 nm. Each cycle lasted 0.064 min. Steady state conditions were measured for 10 min before addition of PGE\(_2\) (0.5 \(\mu\)M, suspended in dimethyl sulfoxide) or LaCl\(_3\) (1 \(\mu\)M). At the end of the measurement period 1 \(\mu\)M 4Br-A23187 (nonfluorescent analog of the Ca\(^{2+}\) ionophore A23187; Molecular Probes, Eugene, OR) was added to determine the maximum response of the cells (data not shown). The data were translated to ASCII format and converted to [Ca\(^{2+}\)]\(\text{free}\), using Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA). [Ca\(^{2+}\)]\(\text{free}\) was calculated using the method described by Grynkiewicz et al. (15) and corrected for pH effects on K\(_{DA}\) of fura-2 as described by Martinez-Zagallan et al. (16) using the formula:

\[
[\text{Ca}\^{2+}]_{\text{free}} = \frac{K_{DA}}{(R - R_{\infty})/R_{\infty} - R)}
\]

where K\(_{DA}\) is the apparent dissociation constant of fura-2 for Ca\(^{2+}\) (140 nM), R\(_{\infty}\) is determined when fura-2 is completely quenched (0.4783) and represents the minimum fluorescence 340/380 nm ratio; R\(_{\infty}\) is the 340/380 nm ratio when fura-2 is saturated with Ca\(^{2+}\) (5.079), and R is the measured fluorescence 340/380 nm ratio. The K\(_{DA}\) (140 nM), R\(_{\infty}\) (5.079), and R\(_{\infty}\) (0.4783) were determined from in situ calibration of fura-2 as described previously (17).

Cell incubations for progesterone determination

Suspensions of large cells (1 x 10\(^6\)/ml) or small cells (5 x 10\(^6\)/ml) were aliquoted to glass culture tubes (12 x 75 mm) containing 1 ml Hanks' or MH medium (0.1% BSA, pH 7.15, 37 \(^\circ\)C) and were incubated for 2 h under atmospheric conditions with continuous shaking. After incubation, cells were pelleted by centrifugation (900 x g, 4 min, 22 \(^\circ\)C) and media were collected for measurement of progesterone content by RIA (14). The pelleted cells were immediately resuspended in Medium 199 for determination of cellular viability as assessed by Trypan blue dye exclusion.

Statistical analysis

Corpora lutea from one to three animals were pooled for preparation of cells. Each experiment (n) represents a different tissue preparation. Data are expressed in the text as the mean \(\pm\) SEM. Statistical significance between multiple treatments were determined by analysis of variance followed, where appropriate, by Student-Newman-Kuels multiple range testing. Differences between two means were determined by Student's t test for paired data. Significance was assigned at the 0.05 level.

Data from the recovery phase of the PGE\(_2\)-induced [Ca\(^{2+}\)]\(\text{free}\) transient were fit to compartmental models using nonlinear regression techniques. The number, size, and rate constants of the defined compartments were estimated using the Simplex method as previously described (7; MINSP, Micromath Scientific Software, Salt Lake City, UT).
Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Fluorophores were obtained from Molecular Probes (Eugene, OR). [3H]Progestrone was obtained from New England Nuclear Research Products (Boston, MA). The antibody to progesterone, GDN-237, was kindly provided by Dr. G. B. Niwender.

Results

The dependence of the PGF-a-induced calcium transient on extracellular calcium was examined by incubation of large cells in Ca2+-free Hanks' medium (plus or minus 1 mM EGTA). A ten-min exposure of large cells to Ca2+-free Hanks' (+EGTA) reduced (P < 0.05) steady state [Ca2+]I (Table 1, Fig. 1). Subsequent addition of PGF2α (0.5 μM) initiated a transient increase in [Ca2+]I, the magnitude of which was reduced (P < 0.05) compared to the transient observed in complete Hanks' medium (Table 1 and Fig. 1). In addition, unlike incubations in complete Hanks', the [Ca2+]I levels returned to basal levels within 3 min (Table 1 and Fig. 1).

Incubation of large cells in Ca2+-free Hanks' (-EGTA) did not significantly decrease resting [Ca2+]I levels. The size of the PGF2α-induced transient was reduced (P < 0.05) compared with that in complete Hanks', however it was greater (P < 0.05) than the transient in Ca2+-free (+EGTA; Table 1 and Fig. 1). In addition, unlike incubations in complete Hanks', [Ca2+]I levels returned to pretreatment [Ca2+]I levels within 3 min (Table 1, Fig. 1).

Resting [Ca2+]I levels in large cells were reduced by incubation in medium in which NaH2PO4, NaHCO3, and MgSO4 were removed from complete Hanks (modified Hanks, MH medium). Modified Hanks' medium (buffered with HEPES) was used as the incubation medium whenever LaCl3 was added since a precipitate formed when LaCl3 was added to complete Hanks'. Basal secretion of progesterone and cellular viability were not modified by incubation in MH medium (data not shown). However, resting [Ca2+]I levels and the PGF2α-induced calcium transient were reduced (P < 0.05) in MH medium compared to complete Hanks' (Table 1, Fig. 2, and see Fig. 5a).

The recovery phase of the PGF2α-induced calcium transient was best described by a two compartment model under all incubation conditions used in the present study. The size of the rapidly exchanging compartment was reduced (P < 0.05) in Ca2+-free Hanks' (+ or -EGTA) and MH medium (Table 1). Whereas, incubation in Ca2+-free medium (plus EGTA) was the only condition that reduced the size of the slowly exchanging compartment. The rate constant of the rapidly exchanging compartment was reduced (P < 0.05) in Ca2+-free medium (plus EGTA) and the rate constant of the slowly exchanging compartment was increased (P < 0.05) in Ca2+-free medium (+ or -EGTA; Table 1). Modified Hanks' medium (which contained calcium) did not affect the rate constants of either compartment.

Prolonged incubation (10 min) of large cells in 1 mM LaCl3 resulted in an increase (P < 0.05) in resting [Ca2+]I levels (see Fig. 5a). Addition of 0.5 μM PGF2α also produced a sustained elevation (P < 0.05) of [Ca2+]I. (102 ± 10 nM, n = 5, Fig. 1, and see Fig. 5a). Lanthanum affected the response of large cells to PGF2α in two ways. First, preincubation of large cells with LaCl3 (2 min) completely prevented the PGF2α-induced calcium transient (data not shown). Whereas, simultaneous addition of PGF2α and LaCl3 resulted in a rapid and marked increase in [Ca2+]I, that was sustained throughout the remainder of the measurement period (Fig. 3).

The inhibitory effect of PGF2α on secretion of progesterone by these cells was not altered by pretreatment with LaCl3 (2 min) (data not shown).

Table 1. Comparison of PGF2α-induced calcium transient parameters in complete Hanks vs. Ca2+-free (+EGTA) Hanks' and Modified Hanks'

<table>
<thead>
<tr>
<th></th>
<th>Complete Hanks</th>
<th>Modified Hanks</th>
<th>Ca2+-free (+EGTA)</th>
<th>Ca2+-free (+EGTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state [Ca2+]I (nM)</td>
<td>62 ± 5 (5)</td>
<td>50 ± 2 (12)*</td>
<td>50 ± 4 (3)</td>
<td>27 ± 4 (4)*</td>
</tr>
<tr>
<td>Size of PGF2α calcium transient (nM increase)</td>
<td>87 ± 2 (5)*</td>
<td>32 ± 6 (4)**</td>
<td>29 ± 5 (12)**</td>
<td>10 ± 1 (4)**</td>
</tr>
<tr>
<td>Post transient [Ca2+]I (nM above steady state [Ca2+]I)</td>
<td>37 ± 2 (5)*</td>
<td>9 ± 3 (4)*</td>
<td>6 ± 1 (2)*</td>
<td>2 ± 1 (4)</td>
</tr>
<tr>
<td>Rapidly exchanging compartment</td>
<td>62 ± 10 (4)</td>
<td>22 ± 4 (3)*</td>
<td>21 ± 3 (3)*</td>
<td>13 ± 3 (5)*</td>
</tr>
<tr>
<td>Size (nM)</td>
<td>3.6 ± 0.7</td>
<td>6.6 ± 3</td>
<td>4.6 ± 0.4</td>
<td>1.8 ± 0.1*</td>
</tr>
<tr>
<td>Rate constant (1/min)</td>
<td>140 ± 9</td>
<td>187 ± 40</td>
<td>102 ± 19</td>
<td>47 ± 11*</td>
</tr>
<tr>
<td>Slowly exchanging compartment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size (nM)</td>
<td>0.017 ± 0.002*</td>
<td>0.023 ± 0.002</td>
<td>0.047 ± 0.002*</td>
<td>0.037 ± 0.001*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE; (n) = number of experiments. The values were calculated by fitting data from the recovery phase of the PGF2α-induced [Ca2+]I transient, as described in Materials and Methods.
* Different from complete Hanks'.
** Different from steady state [Ca2+]I (P < 0.05).
was also decreased in a sustained increase in gestrone secretion (Fig. 5a). Secretion of progesterone was not altered by small cells incubated in Ca\(^{2+}\)-free Hanks’ (plus EGTA) and appeared to be reduced in the one experiment with 5 μM BAPTA (Fig. 5d). Secretion of progesterone by small cells was not altered by Ca\(^{2+}\)-free medium (−EGTA), LaCl\(_3\) or PGF\(_{2\alpha}\) (Fig. 5d). Small cell viability was not affected by any of the incubation conditions (data not shown).

Discussion

The contribution of extracellular calcium to the PGF\(_{2\alpha}\)-induced calcium response was investigated by incubation of ovine large cells in Ca\(^{2+}\)-free medium (+ or −1 mM EGTA). Incubation of large luteal cells in Ca\(^{2+}\)-free medium (+EGTA) reduced basal [Ca\(^{2+}\)]\(_i\), and the PGF\(_{2\alpha}\)-induced transient increase in [Ca\(^{2+}\)]\(_i\). This observation provides evidence that both extracellular and intracellular calcium contribute to the PGF\(_{2\alpha}\)-induced transient increase in [Ca\(^{2+}\)]\(_i\) in ovine large cells. A PGF\(_{2\alpha}\)-induced calcium transient has been observed in several types of luteal cells, however a contribution of extracellular Ca\(^{2+}\) to the response has not been consistently observed. In Ca\(^{2+}\)-free medium, the PGF\(_{2\alpha}\)-induced transient increase in [Ca\(^{2+}\)]\(_i\) was not affected in suspensions of rat luteal cells (9), was attenuated in bovine large
changes in the sizes of the rapidly and slowly exchanging compartments could also reflect two calcium transport compartments the rapidly and slowly exchanging compartments could be due to changes in the capacities of transport mechanisms that work to maintain calcium homeostasis. The distinction between two compartments or two transporters could not be made in these studies.

Incubation of large cells in Ca\(^{2+}\)-free medium without exposure of the cells to Ca\(^{2+}\)-free conditions and differing methods of measuring [Ca\(^{2+}\)]\(_i\) may have contributed to these varied results.

An attenuated PGF\(_{2\alpha}\)-induced calcium transient produced by cells incubated under Ca\(^{2+}\)-free conditions suggests a direct contribution of extracellular calcium to the transient. However, an alternative explanation may be that exposure of the cells to Ca\(^{2+}\)-free medium depletes intracellular calcium stores. This depletion would decrease the amount of intracellular calcium available for release by PGF\(_{2\alpha}\). This appeared to be the case in rat luteal cells in which addition of EGTA just before addition of PGF\(_{2\alpha}\) did not alter the PGF\(_{2\alpha}\)-induced calcium transient, whereas preincubation of the cells with EGTA and ionomycin completely eliminated the PGF\(_{2\alpha}\)-induced transient (9). In the present study, large cells preincubated in Ca\(^{2+}\)-free medium (+EGTA) for 10 min demonstrated a reduction in resting [Ca\(^{2+}\)], and the size of both the rapidly- and slowly exchanging calcium compartments. These reductions suggest a depletion of intracellular calcium stores. In addition to distinct Ca\(^{2+}\) containing compartments the rapidly and slowly exchanging compartments could also reflect two calcium transport mechanisms acting on one calcium compartment. Thus, changes in the sizes of the rapidly and slowly exchanging compartments could be due to changes in the capacities of transport mechanisms that work to maintain calcium homeostasis. The distinction between two compartments or two transporters could not be made in these studies.

Incubation of large cells in Ca\(^{2+}\)-free medium without EGTA did not reduce steady state [Ca\(^{2+}\)]\(_i\), levels. However, the PGF\(_{2\alpha}\)-induced calcium transient was attenuated and the size of the rapidly exchanging calcium compartment was reduced. Taken together, the results of incubations of cells in Ca\(^{2+}\)-free medium (+ or -EGTA) suggest that mobilization of Ca\(^{2+}\) from intracellular stores is primarily responsible for the PGF\(_{2\alpha}\)-induced calcium transient, and the magnitude of the transient appears to be dependent on the amount of calcium available in the rapidly exchanging calcium pool.

The PGF\(_{2\alpha}\)-induced calcium transient was modified by removal of phosphate and carbonate compounds from Hanks’ medium (MH medium). Although CaCl\(_2\) (1.67 mM) was present in MH medium, steady state [Ca\(^{2+}\)]\(_i\) in the PGF\(_{2\alpha}\)-induced calcium transient was modified by the inorganic calcium antagonist, LaCl\(_3\). Lanthanum displaces calcium from binding sites on the external surface of the plasma membrane and inhibits calcium efflux and influx in many cell types (18–20), including large and small ovine luteal cells (Wegner, J. A., R. Martinez-Zaguilan, R. J. Gillies, and P. B. Hoyer, submitted for publication). Preincubation with LaCl\(_3\) completely inhibited the PGF\(_{2\alpha}\)-induced calcium transient (data not shown). This observation suggests an absolute requirement of calcium influx for the PGF\(_{2\alpha}\)-induced calcium transient. However, this finding is not entirely consistent with the results obtained from the...
Fig. 5. Effect of incubation medium on steady state [Ca$^{2+}$], and basal secretion of progesterone in ovine large (A, B) and small (C, D) luteal cells. Suspensions of large ($5 \times 10^6$ cells/ml) or small ($1 \times 10^6$ cells/ml) cells were loaded with fura-2 and its fluorescence was recorded. [Ca$^{2+}$], was calculated using Eq [I] as described in Materials and Methods. Steady state [Ca$^{2+}$], levels were determined from the fluorescence after a 10-min exposure to the treatment condition. The data are expressed as mean ± SE, (n) = number of separate experiments for each treatment condition. Progesterone was measured in the medium after a 2-h incubation of large ($1 \times 10^6$ cells/ml) or small ($5 \times 10^6$ cells/ml) cells as described in Materials and Methods. The data are expressed as mean ± SE % control (1.67 mM CaCl$_2$, no treatment) and values of each (n) represent mean of triplicate incubations. Statistical analysis was performed using the absolute progesterone values. All treatments, except those containing LaCl$_3$, (MH medium) were performed in Hanks’ medium. N.D. = Not determined. * Different from treatment control value (P < 0.05).

Ca$^{2+}$-free medium (+EGTA). Thus, a LaCl$_3$-displaceable calcium pool may be required to initiate the PGF$_{2 \alpha}$-induced calcium transient in large luteal cells. Attenuation of a hormone-induced calcium transient by LaCl$_3$ has been reported in rat pancreatic acini (18). That result prompted the authors to propose that mobilization of intracellular calcium may partly depend on the availability of a La$^{3+}$-sensitive pool of cell surface calcium.

Other possible explanations for the elimination of the PGF$_{2 \alpha}$-induced transient by preincubation with LaCl$_3$ include 1) an interference of LaCl$_3$ with the binding of PGF$_{2 \alpha}$, to the receptor, 2) an inhibitory effect of LaCl$_3$, either directly or indirectly, on membrane bound reactions associated with the PGF$_{2 \alpha}$ response, such as the generation of inositol trisphosphate and/or 3) influx of LaCl$_3$ into the cell which inhibits calcium release from intracellular calcium stores. Interference of LaCl$_3$ with the binding of PGF$_{2 \alpha}$ to its receptor or generation of inositol trisphosphate seems unlikely since when PGF$_{2 \alpha}$ and LaCl$_3$ were added simultaneously, a rapid increase in [Ca$^{2+}$], similar to the increase observed with PGF$_{2 \alpha}$ alone, was demonstrated. Although LaCl$_3$ acts primarily at the external surface of the plasma membrane, LaCl$_3$ has been shown to enter cultured myocardial cells after prolonged incubation (>60 min; 21) or after sarcolemma damage due to ischemia (22). In the present study the PGF$_{2 \alpha}$-induced calcium transient was eliminated after only a 2-min exposure to LaCl$_3$. In addition, previous experiments with ovine large and small luteal cells incubated with LaCl$_3$ strongly suggest that LaCl$_3$ does not cross the plasma membrane within the time frame of the experiments performed (Wegner, J. A., R. Martinez-Zaguilan, R. J. Gillies, and P. B. Hoyer, submitted for publication). Alterations of intracellular calcium compartments due to changes in a La$^{3+}$-sensitive pool of cell surface calcium cannot be ruled out in the present
experiments.

In summary, the transient response of PGF₂α was altered in luteal cells incubated in Ca²⁺-free medium (plus or minus EGTA) and MH medium. After the PGF₂α-induced increase, [Ca²⁺]∗ returned to 7%, 12%, 18%, and 60% above steady state in Ca²⁺-free (plus EGTA), Ca²⁺-free (−EGTA), MH, and complete Hanks' medium, respectively. Since the slowly exchanging compartment was not significantly reduced in Ca²⁺-free medium (−EGTA) or in MH medium, it appears that maintenance of the sustained elevation in [Ca²⁺]∗ in response to PGF₂α is dependent upon extracellular calcium and/or the size of the rapidly exchanging compartment. Thus, a second contribution of extracellular Ca²⁺ to the PGF₂α-induced calcium response may be to supply calcium during the posttransient phase of the PGF₂α-induced calcium response. A dependence of the sustained increase in [Ca²⁺]∗ on extracellular calcium has also been demonstrated in rat and bovine luteal cells (9, 17).

A relationship between the ability of PGF₂α to induce an increase in [Ca²⁺]∗, and an inhibition of secretion of progesterone was demonstrated in this study. The inhibitory effect of PGF₂α on secretion of progesterone was reduced under conditions that reduced the magnitude of the PGF₂α-induced calcium transient. A more detailed experimental design and an improved understanding of cellular events regulating progesterone synthesis are required before a quantitative correlation between the PGF₂α-induced calcium transient and progesterone secretion can be demonstrated.

The effect of steady state [Ca²⁺]∗, levels on secretion of progesterone in large and small cells was also examined. Secretion of progesterone and steady state [Ca²⁺]∗, were reduced by incubation of large and small luteal cells in Ca²⁺-free medium (+EGTA) or 5 μM BAPTA. These results suggest that a minimum [Ca²⁺]∗, level is required to maintain basal secretion of progesterone in both cell types. Removal of extracellular calcium did not decrease basal progesterone secretion or cAMP levels in rat luteal cells (6, 23). However, LH-stimulated secretion of progesterone was inhibited in calcium-free medium obtained from perfused ovine luteal slices (24). Since [Ca²⁺]∗, levels were not directly measured in those studies, the extent of reduction in steady state [Ca²⁺]∗, is not known. Cytosolic-free calcium levels were reduced by BAPTA in rat luteal cells, but the effect of BAPTA on basal secretion of progesterone was not reported (6).

In the present study, [Ca²⁺]∗, was increased in ovine large, but not small luteal cells, by incubation with LaCl₃ and PGF₂α. In addition, both LaCl₃ and PGF₂α inhibited secretion of progesterone in large, but not small cells. Thus, there appears to be a relationship between sustained elevations of [Ca²⁺]∗, and inhibition of progesterone secretion in large cells. This relationship is further supported by studies using the calcium ionophore, A23187 (1 μM), in which it was shown to inhibit basal progesterone secretion in large, but not small luteal cells (3). A dissimilarity between ovine large and small luteal cells in regulating [Ca²⁺]∗, levels is suggested in a study by Wiltbank et al. (8) in which [Ca²⁺]∗, readily returned to basal levels within 3 min after the addition of 1 μM A23187 in small cells, but resulted in a sustained (up to 5 min) elevation of [Ca²⁺]∗, in large. The tendency of small cells to return to steady state [Ca²⁺]∗, levels may explain why inhibition of progesterone secretion by A23187 was not observed in small cells under conditions that caused inhibition in large (3). It has also been proposed that a critical level of [Ca²⁺]∗, may be required for inhibition of LH-stimulated progesterone secretion in bovine large luteal cells (10).

Basal secretion of progesterone was inhibited by a sustained decrease or increase in [Ca²⁺]∗, in ovine large luteal cells. This provides evidence that optimal secretion of progesterone may require a specific range of [Ca²⁺]∗, within these cells. Key enzymes in the progesterone synthetic pathway may not be adequately stimulated at reduced [Ca²⁺]∗, levels, whereas sustained elevations in [Ca²⁺]∗, levels may inhibit secretion of progesterone due to cytotoxic effects of calcium on a variety of cellular reactions. A sustained elevation of [Ca²⁺]∗, has been shown to have deleterious effects in myocardial cells (11, 12) and hepatocytes (13). Sustained elevations in [Ca²⁺]∗, may result in redistribution of calcium within intracellular compartments and/or alter membrane properties. Such alterations could subsequently inhibit cell function and/or cell viability. In the luteal cell, high levels of [Ca²⁺]∗, may negatively affect progesterone secretion by inhibiting delivery of cholesterol to the mitochondria (25). Furthermore, elevated [Ca²⁺]∗, may result in increased mitochondrial free Ca²⁺ that may interfere with ATP production (6) and/or cholesterol to pregnenolone conversion (26, 27). A decrease in membrane fluidity of regressing rat luteal cells has been attributed to stimulation of a Ca²⁺-mediated pathway (28). Structural changes in cell membranes may alter calcium transport and alter the ability of cells to regulate [Ca²⁺]∗, (11, 12).

Maintenance of sustained elevations in [Ca²⁺]∗, levels may also be involved in biochemical reactions initiated by a transient increase in calcium, such as activation of protein kinase C. Phorbol ester stimulated protein kinase C activity and inhibited progesterone secretion in ovine large luteal cells (2, 29). Thus, maintenance of elevated [Ca²⁺]∗, level may play a role in activation of second messenger pathways for mediating intracellular induction of luteal regression.

The results presented here suggest that a critical range of [Ca²⁺]∗, may regulate progesterone production in ovine large luteal cells. This range may represent an optimal
window of calcium concentration that is required for maintenance of proper luteal function before initiation of luteolysis. Exceeding this range, by stimulation with PGF₂α, may trigger events involved in functional as well as structural phases of luteolysis.

In summary, the results of this study demonstrated that the PGF₂α-induced calcium transient can be modified by alterations in the availability of extracellular calcium. More specifically, a reduction in the size of a rapidly exchanging calcium compartment appears to be responsible for attenuation of the PGF₂α-induced calcium transient. Although the rapidly exchanging compartment was not specifically identified, the size of the compartment appears to be dependent upon extracellular calcium and/or the presence of phosphate and carbonate compounds in the medium. Additionally, a relationship between the ability of PGF₂α to produce an increase in [Ca²⁺], and an inhibition of progesterone secretion in large cells was observed. These results provide further evidence that in the ewe the luteolytic effects of PGF₂α are mediated in the large cell by the ability of this hormone to produce elevations in cytosolic-free calcium. Future studies will determine the role of calcium-dependent second messenger pathways in this induction of luteal regression.

Acknowledgments

The authors wish to thank Mr. S. L. Marion, Mr. W. Engleberg, and Ms. G. M. Martinez for technical assistance.

References

4. Willbank MC, Diskin MG, Flores JA, Niwender GD 1990 Regulation of the corpus luteum by protein kinase C. II. Inhibition of prostaglandin F₂α-induced pregnenolone metabolism to progesterone in bovine luteal cells. Endocrinology 125:144-151
22. Hoyer PB, Kong W 1989 Protein kinase A and C activities and endogenous substrates in ovine small and large luteal cells. Mol Cell Endocrinol 62:203-216
SECTION 4.

Differing effects of LaCl$_3$ on cytosolic free calcium in ovine large and small luteal cells.

DIFFERING EFFECTS OF LaCl₃ ON CYTOSOLIC FREE CALCIUM LEVELS IN OVINE LARGE AND SMALL LUTEAL CELLS

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SUMMARY

The effect of the calcium antagonist, LaCl₃, on cytosolic free calcium levels ([Ca²⁺]ᵢ) in ovine large and small luteal cells was examined. Calcium (⁴⁵Ca²⁺) efflux and uptake were both inhibited (P < 0.05) by LaCl₃ in large and small cells. Addition of 1 mM LaCl₃ to a suspension of large cells loaded with FURA-2 caused a gradual increase in [Ca²⁺]ᵢ, which was 79 ± 19 nM (n=4; P < 0.05) above steady state levels after a 10 min incubation. However, [Ca²⁺]ᵢ levels were not altered at any time in small cells incubated with LaCl₃. Control studies were conducted to determine whether the La³⁺-induced increase in fluorescence
in large cells was due to a direct interaction of $\text{La}^{3+}$ with FURA-2 rather than a true increase in $[\text{Ca}^{2+}]$. Those results support that was not the case. Therefore the results presented here suggest that ovine large and small luteal cells differ in the regulation of cytosolic free calcium levels.
INTRODUCTION.

The corpus luteum produces the steroid hormone progesterone, which is absolutely required for successful reproduction. During the estrous cycle the corpus luteum is formed on the ovary and undergoes regression at the end of the luteal phase if pregnancy does not occur. The signal for regression (i.e., luteolysis) in many species, including the ewe, is prostaglandin \( \text{F}_2\alpha \) (PGF\(_{2\alpha}\); McCracken et al., 1970).

Two morphologically and functionally distinct steroidogenic cell types, designated small (12-22 \( \mu \)m diameter) and large (> 22 \( \mu \)m diameter), have been identified in the corpus luteum of many species, including the ewe (Fitz et al., 1982; Rodgers and O'Shea, 1982; Rodgers et al., 1985). Both cell types secrete progesterone, but differ in many aspects of hormonal regulation of steroidogenesis (Hoyer and Niswender, 1985). Recent evidence suggests that in the ewe, direct cellular effects of PGF\(_{2\alpha}\) are mediated by large, rather than small cells (Balapure et al., 1989). Initiation of regression at the cellular level appears to result from a PGF\(_{2\alpha}\)-induced increase in \([\text{Ca}^{2+}]_i\) followed by a sustained elevation of \([\text{Ca}^{2+}]_i\), which is specifically observed in large cells (Hoyer et al., 1990a,b; Wiltbank et al., 1989; Wegner et al., 1990). These findings suggest that PGF\(_{2\alpha}\)-induced luteal regression is mediated by intracellular \( \text{Ca}^{2+} \)-dependent pathway(s).
In most tissues maintenance of calcium homeostasis is important for basic cellular function and optimal responsiveness to hormonal stimulation. The role of extracellular calcium in regulating calcium homeostasis can be assessed by removal of extracellular calcium or inhibition of transmembrane calcium flux by calcium antagonists such as LaCl₃. Lanthanum (La³⁺) is similar in size to that of the calcium ion (Ca²⁺) and displaces cell surface Ca²⁺ without causing structural modifications (Weiss, 1974). Lanthanum has been demonstrated to be confined to the extracellular space in healthy cells (Kwan and Putney, 1990; Weiss, 1974). Thus, La³⁺ is a valuable tool for studying calcium metabolism, especially with respect to the study of transmembrane movement of calcium in cells.

Since La³⁺ competes for Ca²⁺ binding sites, it might be expected to interfere with the measurement of [Ca²⁺]ᵢ by FURA-2, a fluorescent dye that complexes with Ca²⁺. Displacement of FURA-2-bound Ca²⁺ by La³⁺ might either quench FURA-2 fluorescence or cause fluorescent changes similar to those produced by Ca²⁺ binding. In view of the ability of PGF₂α to induce a sustained increase in [Ca²⁺]ᵢ in large, but not small cells, the present study was designed to compare the effect of LaCl₃ on steady state [Ca²⁺]ᵢ levels in the two cell types. Determining this required a careful investigation of whether the observed changes in [Ca²⁺]ᵢ levels with LaCl₃ treatment are due to increases in [Ca²⁺]ᵢ or to direct interactions of La³⁺ with FURA-2.
MATERIALS AND METHODS.

Animals. Western range ewes were synchronized with injections of PGF$_{2\alpha}$ (i.m., 10 mg, Lutalyse; Upjohn Company, Kalamazoo, MI, USA) on days -12 and -3. Follicular development was induced with a subcutaneous injection of pregnant mares' serum (1000 IU, s.c., Day -3) followed (day 0) with an injection of human chorionic gonadotropin (750 IU, i.v., Sigma Chemical Co., St. Louis, MO, USA). The experiments to be performed required large numbers of separated fractions of small and large luteal cells. Therefore, this treatment protocol was utilized to induce multiple ovulations so that sufficient numbers of cells could be recovered (Hild-Petito et al., 1987).

Tissue collection. Corpora lutea were surgically collected on day 10 from superovulated western range ewes as described by Hild-Petito et al. (1987). Corpora lutea were pooled and placed in sterile Medium 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with (g/l): 0.35 NaHCO$_3$, 4.7 Hepes, 1.0 BSA, 0.079 penicillin G, 0.1 streptomycin sulphate, 0.05 neomycin sulphate, pH 7.35 for transport to the laboratory.

Tissue preparation. Corpora lutea were decapsulated, sliced and enzymatically dissociated into single cell suspensions (0.4% collagenase : Worthington
Biochemical, Malvern, PA, USA; 0.0025% DNAse: Sigma; complete Hanks; pH 7.35, Hild-Petitio et al., 1987). Dissociation proceeded by incubation, 3-5 hr, in a shaking water bath at 37°C until visible clumps disappeared. Single cell suspensions were separated into large and small cell fractions by elutriation as previously described (Hild-Petitio et al., 1987). Typically the small cell fraction (12-22 μm) was free of large cells and the large cell fraction (> 22 μm) contained 10-20% small cells by number. Following elutriation, viability was determined by trypan blue dye exclusion and typically found to be >90% in small cells and >75% in large. Cellular viability was not altered in small or large cells following 2 hrs incubation with LaCl₃. Furthermore, viability was maintained during incubation (30 min) of both cell types with FURA-2 and LaCl₃ (1 mM).

**Intracellular calcium measurements.** Aliquots of large (5 x 10⁵/3 ml) or small (1 x 10⁶/3 ml) cells were washed once with modified Hanks medium (MH medium: 140 mM NaCl, 5 mM KCl, 1.67 mM CaCl₂, 10 mM Hepes, 0.8 mM MgCl₂, 0.1% BSA, 1 mM glucose, pH 7.15, 37°C) and incubated (30 min at 37°C in 5% CO₂ atmosphere) in 3 ml MH medium containing 2 μM FURA-2 (AM). In experiments involving simultaneous [Ca²⁺]ᵢ and pH measurements, cells were preloaded with both 2 μM FURA-2 (AM) and 20 μM SNARF-1(AM). In experiments involving chelation of [Ca²⁺]ᵢ, cells were preloaded with both 2 μM FURA-2 (AM) and 20 μM BAPTA(AM)(5,5'-dimethylbis-(o-aminophenoxy)ethane-N,N,N’N'-tetraaceticacid). At the end of dye loading, cells were washed, resuspended (5 ml MH medium),
and re-incubated (30 min, 37°C) to allow complete hydrolysis of FURA-2 (AM). After the second incubation, cells were washed, resuspended (3 ml MH medium), and transferred to a temperature-controlled, stirring cuvette unit housed in an SLM8000C spectrofluorometer (SLM, Urbana, IL). All experiments were performed at 37°C (Martínez-Zagulán et al., 1991). Microscopic examination demonstrated that the intracellular pattern of FURA-2 distribution in both cell types was diffuse rather than punctate.

Fluorescence measurements from cell suspensions were collected using a continuous acquisition mode (emission at 510 nm, excitation at 340, 360 and 380 nm) with each cycle lasting 3.8 sec. The data acquisition cycle during simultaneous measurement of [Ca^{2+}] and pH lasted 16 sec (emission at 510 nm, excitation at 340, 360 and 380 nm (FURA-2 conditions); followed by excitation at 534 nm and emission at 584, 600, and 644 nm (SNARF-1 conditions)). The 360 nm and 600 nm wavelengths represent the isoexcitation and isoemission points for FURA-2 and SNARF-1, respectively. The isoexcitation or isoemission point is proportional to the total amount of intracellular dye and can be used to evaluate possible quenching and/or other artifacts. Steady state conditions were measured for 10 min before addition of LaCl₃ (1 mM) followed by measurements for an additional 15 min. At the end of the measurement period 1 μM 4Br-A23187 (non-fluorescent analog of the Ca^{2+} ionophore A23187; Molecular Probes,
Eugene, OR, USA) was added to determine the sensitivity of the cell suspension to increased \([\text{Ca}^{2+}]_i\). Data were translated to ASCII format and converted to \([\text{Ca}^{2+}]_i\) using Lotus 1-2-3 (Lotus Development Corp.). Cytosolic-free calcium levels were calculated using the method described by Grynkiewicz et al. (1985), and corrected for pH effects on FURA-2 \(K_{d_a}\) (Martínez-Zaguilán et al., 1991) using:

\[
[\text{Ca}^{2+}]_i = K_{d_a} \times \left(\frac{S_a}{S_b}\right) \times \left(\frac{(R - R_{\text{min}})(R_{\text{max}} - R)}{(R_{\text{max}} - R_{\text{min}})}\right)
\]

where \(K_{d_a}\) is the apparent dissociation constant of FURA-2 for \(\text{Ca}^{2+}\), \(S_a/S_b\) is the ratio of fluorescence intensity at 380 nm at high and low \([\text{Ca}^{2+}]_i\); \(R_{\text{min}}\) is determined when FURA-2 is completely free of \(\text{Ca}^{2+}\) and represents the minimum fluorescence 340/380 nm ratio; \(R_{\text{max}}\) is the 340/380 nm ratio when FURA-2 is saturated with \(\text{Ca}^{2+}\), and \(R\) is the measured fluorescence 340/380 nm ratio. All the \(R\) values were corrected for autofluorescence. The pH-corrected values for \(K_{d_a}\), \(R_{\text{min}}\) and \(R_{\text{max}}\) were determined by in situ calibration of small luteal cells (Wegner et al., 1990).

**Calcium fluxes.** \(^{45}\text{Ca}^{2+}\) efflux: Large (2 x 10^4 cells/0.5 ml) or small (1 x 10^5 cells/0.5 ml) cells were preloaded to isotopic equilibrium with 5 μCi/ml \(^{45}\text{Ca}^{2+}\) (17.2 mCi/mg, New England Nuclear, Boston, MA) in MH medium (90 min, 37°C,
pH 7.15) with continuous shaking (New Brunswick metabolic shaker). After loading, cells were collected in pre-cooled plastic tubes, centrifuged for 1 min at 100 x g, washed in 3 ml MH buffer (4°C) and centrifuged (1 min). Cells were resuspended in an appropriate volume of MH buffer at 37°C. A 20 µl aliquot of the cell suspension was immediately added to 0.5 ml MH medium (control) for determination of ⁴⁵Ca²⁺ content at time 0. Remaining cells were aliquoted to tubes containing 0.5 ml MH medium ± LaCl₃ (1 mM) and incubated (duplicates) for the specified time periods (3-15 min). The reaction was stopped by diluting the 0.5 ml cell suspension in 10 ml of wash solution (154 mM NaCl, 4°C), followed by rapid filtration through Nucleopore filters (25 µm). Filters were rinsed once with 10 ml wash solution (4°C) and placed in 4 ml scintillation fluid and the samples counted. Data were calculated as percent radioactivity remaining in cells compared with radioactivity present at time 0 (Williams et al., 1978).

⁴⁵Ca²⁺ uptake: Calcium uptake was initiated by addition (20 µl) of large (2 x 10⁴ cells/0.5 ml) or small (1 x 10⁵ cells/0.5 ml) cells to tubes (duplicates) containing 0.5 ml MH medium (pH 7.15, 37°C) with ⁴⁵Ca²⁺ (5 µCi/ml) with or without LaCl₃ (1 mM). Incubation was stopped at the specified times (0-30 min) and samples were processed as described above. Uptake data were calculated as nmol Ca²⁺/20,000 cells corrected for specific activity of the incubation media (Williams et al., 1978).
Statistical analysis. Corpora lutea from 1-3 animals were pooled in each tissue preparation. Each experiment (n) represents a separate tissue preparation. Data are expressed as mean ± SE. Statistical significance between multiple treatments were determined by analysis of variance followed, where appropriate, by Student-Newman-Kuels multiple range testing. Differences between two means were determined by Student's t-test for paired data. Significance was assigned at the 0.05 level.

Chemicals. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fluoroprobes were obtained from Molecular Probes (Eugene, OR, USA). $^{45}$Ca$^{2+}$ was purchased from New England Nuclear Research Products (Boston, MA, USA).
RESULTS.

**Calcium concentrations and flux.** The importance of extracellular Ca\(^{2+}\) on luteal function (i.e. secretion of progesterone) was initially assessed using the inorganic calcium antagonist, cobalt chloride, CoCl\(_2\). Cobalt (4 mM) inhibited (P < 0.05) progesterone secretion in large (65 ± 7% Control, n=7) and small (79 ± 5% Control, n=6) luteal cells. However, CoCl\(_2\) was observed to quench FURA-2 fluorescence (data not shown; Kwan and Putney, 1990). Thus, another calcium antagonist, LaCl\(_3\) was selected. Lanthanum inhibited progesterone production in large cells (65 ± 3% Control, n=13) to the same extent as CoCl\(_2\) and did not appear to cause significant quenching of intracellular FURA-2 fluorescence.

The addition of LaCl\(_3\) to Hanks medium resulted in the formation of a precipitate. Thus, the use of LaCl\(_3\) required removal of phosphate and carbonate compounds from Hanks medium (modified Hanks medium, MH medium). Resting [Ca\(^{2+}\)]\(_i\) levels were reduced in large cells incubated in MH medium, however there was no significant effect of the medium on basal or PGF\(_{2\alpha}\)-inhibited progesterone production (Wegner et al., 1991). Addition of LaCl\(_3\) (1 mM) to suspensions of large cells loaded with FURA-2 caused a gradual increase in FURA-2 fluorescence apparently due to an increase in [Ca\(^{2+}\)]\(_i\) (Figure 1A, Table 1). Initially [Ca\(^{2+}\)]\(_i\) was
increased \( 9 \pm 3 \) nM \((n=4)\) above steady state levels. After 10 min, \([\text{Ca}^{2+}]_i\)
levels were increased \((P < 0.05)\) to \(79 \pm 19\) nM \((n=4)\) above pre-treatment
levels. Both \(45^{\text{Ca}}\) uptake and efflux were inhibited by \(\text{LaCl}_3\) in large cells (Figure
2A, 2B). Although \(\text{LaCl}_3\) also inhibited \(45^{\text{Ca}}\) uptake and efflux in small cells
(Figure 3A, 3B), no increase in \([\text{Ca}^{2+}]_i\) was observed in small cells (Figure 1C,
Table 1).

**Control studies.** Lanthanum antagonizes the action of \(\text{Ca}^{2+}\) by displacement of
\(\text{Ca}^{2+}\) from \(\text{Ca}^{2+}\) binding sites (Langer and Frank, 1972). There was, therefore, a
possibility that the apparent increase in \([\text{Ca}^{2+}]_i\) in large cells was due to \(\text{La}^{3+}\)
binding directly to FURA-2 with subsequent alteration of FURA-2 fluorescence.
To determine the effect of \(\text{LaCl}_3\) on FURA-2 fluorescence, the free acid form of
FURA-2, FURA-2(FA), was dissolved \((1 \mu\text{M})\) in \(\text{Ca}^{2+}\)-free MH medium without cells.

Addition of \(1 \mu\text{M} \text{LaCl}_3\), in the absence of cells, resulted in an immediate increase
in the \(340/380\) nm fluorescence ratio (Figure 4A). Examination of the individual
excitation wavelengths \((340, 380\) and \(360\) nm) showed that the increased \(340/380\)
nm ratio was due to an increase in \(340\) fluorescence and a decrease in \(380\) nm
fluorescence (Figure 4B). Furthermore, lanthanum decreased fluorescence
intensity at \(360\) nm, the isoexcitation wavelength of FURA-2. This demonstrates
a quenching effect of \(\text{LaCl}_3\) on FURA-2 fluorescence. These results provide
evidence that \(\text{La}^{3+}\) significantly affects FURA-2 fluorescence in \(\text{Ca}^{2+}\)-free medium.
FIGURE 1. Effect of LaCl₃ on [Ca²⁺]ᵢ in ovine large and small luteal cells. (A) Suspensions of large (5 x 10⁶/3 ml) or (C) small (1 x 10⁶/3 ml) luteal cells were loaded with 2 μM FURA-2. Fluorescence was continuously measured, with addition of LaCl₃ (1 mM) where indicated. [Control, (..........),LaCl₃, (______)]. The resulting 340/380 nm ratios were fit to equation [1] as described in Materials and Methods. Data is representative of 3 experiments. (B and D) Recordings of FURA-2 fluorescence (from A and C, respectively) at the excitation wavelengths of 340, 360 and 380 nm using an emission wavelength of 510 nm. (a.u.) = arbitrary units.
FIGURE 2. The effect of LaCl₃ on calcium efflux and uptake in large luteal cells. (A) Large (2 x 10⁴/0.5 ml) luteal cells were pre-incubated in control medium containing ⁴⁵Ca²⁺ (5 μCi/ml). Efflux was initiated by addition of cells (duplicates) to medium ± 1 mM LaCl₃. Efflux was terminated by rapid filtration and the radioactivity remaining in the cells was counted. Efflux is presented as % ⁴⁵Ca²⁺ remaining in the cells at each time point compared with the cellular content of ⁴⁵Ca²⁺ at t = 0 min. (B) ⁴⁵Ca²⁺ uptake in large luteal cells was initiated by addition of cells (2 x 10⁴/0.5 ml; duplicates) to medium containing ⁴⁵Ca²⁺ (5 μCi/ml) ± LaCl₃ (1 mM). The cells were processed as in efflux and as described in Materials and Methods; ⁴⁵Ca²⁺ content of the cells was measured. The data are expressed as mean ± SE, n=2 separate efflux and uptake experiments * different from control (P < 0.05).
FIGURE 3. The effect of LaCl₃ on calcium efflux and uptake in small luteal cells. (A) Small (1 x 10⁷/0.5 ml) luteal cells were pre-incubated in control medium containing ⁴⁵Ca²⁺ (5 µCi/ml). Efflux was initiated by addition of cells (duplicates) to medium ± 1 mM LaCl₃. Efflux was measured as described in Figure 58. (B) ⁴⁵Ca²⁺ uptake in small luteal cells was initiated by addition of cells (1 x 10⁷/0.5 ml; duplicates) to medium containing ⁴⁵Ca²⁺ (5 µCi/ml) ± LaCl₃ (1 mM). Uptake was measured as described in Figure 58. The data are expressed as mean ± SE, n=2 separate experiments. * different from control (P < 0.05).
at all excitation wavelengths critical to the measurement of [Ca\(^{2+}\)].

A direct interaction of La\(^{3+}\) with FURA-2 can therefore be observed by monitoring 360 nm fluorescence, since fluorescence at this isoexcitation point is unaffected by Ca\(^{2+}\) binding. Figures 1B and 1D show that fluorescence at 360nm was not significantly altered throughout the experimental period with addition of 1 mM LaCl\(_3\) to either large or small cells. Importantly, fluorescence at 340 and 380 nm increase and decrease, respectively in large, but not in small cells. Addition of the calcium ionophore, A23187 (1 or 10 \(\mu\)M), resulted in an increase in 340/380 nm fluorescence, without changing the fluorescence at 360 nm. These data support our contention that LaCl\(_3\) causes a real increase in [Ca\(^{2+}\)]\(_i\) in large cells.

Additional control experiments were performed to establish that the increase in fluorescence in large cells treated with LaCl\(_3\) was due to a true increase in [Ca\(^{2+}\)]\(_i\). In large cells loaded with FURA-2 and the intracellular calcium chelator, BAPTA.AM (20 \(\mu\)M), the effect of LaCl\(_3\) was eliminated (Figure 5). In large cells loaded with FURA-2 and SNARF-1 (pH\(_{in}\)), LaCl\(_3\) induced a gradual increase in [Ca\(^{2+}\)]\(_i\) with no effect on pH\(_{in}\) in large cells (Figure 6). No effect of LaCl\(_3\) on [Ca\(^{2+}\)]\(_i\) or pH\(_{in}\) was observed in small cells (data not shown).

Lanthanum acts primarily at the external surface of the plasma membrane and
under most circumstances does not enter the cell. Thus, LaCl₃ would not be expected to interact with intracellular FURA-2. However, incubation of FURA-2 (AM) loaded large and small cells consistently exhibited a gradual increase in the 340/380 nm ratio with time (Figure 1). The steady rise in FURA-2 fluorescence was attributed to leakage of FURA-2, since addition of probenecid, an anion transporter inhibitor, abolished the increase (McDonough and Burton, 1989; Wegner et al., 1990). Large cells were preincubated with probenecid (2.5 mM) to determine whether the observed La³⁺-induced increase in the 340/380 nm ratio was due to a direct interaction of LaCl₃ with extracellular FURA-2. Probenecid eliminated the gradual increase in fluorescence, however, subsequent addition of LaCl₃ caused a gradual increase (\([\text{Ca}^{2+}\])_t = 5.5 \pm 2 \text{nM/min, } n=2\) in the fluorescent ratio. This value is not different (\(p>0.05\)) from that observed in cells incubated without probenecid (\([\text{Ca}^{2+}\])_t = 8.2 \pm 2 \text{nM/min, } n=4\); Figure 7).
FIGURE 4. Recordings of excitation wavelengths for FURA-2 in Ca^{2+}-free medium in the presence of increasing [LaCl$_3$]. Recordings were obtained in nominally Ca$^{2+}$-free MH medium (without cells) containing 1 μM FURA-2 (FA) and increasing amounts of LaCl$_3$ (1 μM-1 mM) (A) Represents the fluorescence ratio 340/380 nm for FURA-2. (B) Recordings of FURA-2 fluorescence at single excitation wavelengths of 340, 360 (left axis, (-----)) and 380 nm (right axis, (-------)) using emission wavelength of 510 nm (pH 7.15). (a.u.) = arbitrary units.
FIGURE 5. Effect of BAPTA on the LaCl₃-induced calcium response in large luteal cells. Large cells (5 x 10⁶/3 ml) were loaded with 2 μM FURA-2 in the presence of 0 or 20 μM BAPTA as described in Materials and Methods. FURA-2 fluorescence was recorded as described in Figure 57. LaCl₃ (1 mM) was added after an initial 7 min incubation period. Data is representative of 2 experiments.
FIGURE 6. Effect of LaCl₃ on [Ca²⁺]ᵢ and pHᵢ in large luteal cells. Large (5 x 10⁵/3 ml) cells were co-loaded with FURA-2(AM) and SNARF-1(AM). Fluorescence was simultaneously monitored as described in Materials and Methods. The y-axis represents fluorescence ratios of FURA-2 (340/380 nm, [Ca²⁺]ᵢ) and SNARF-1 (644/584, pHᵢ). Data is representative of 2 experiments.
FIGURE 7. The effect of probenecid on the LaCl₃-induced calcium response in large luteal cells. Large (5 x 10⁵/3 ml) luteal cells were loaded with FURA-2 as described in Materials and Methods. Following initial incubation with probenecid (2.5 mM), LaCl₃ (1 mM) was added where indicated. Data is representative of 2 experiments.
DISCUSSION.

Addition of the calcium antagonist, LaCl₃, to ovine large luteal cells resulted in a gradual increase in [Ca²⁺]ᵢ (Figure 1A, B; Table 1). Such an increase in [Ca²⁺]ᵢ could be achieved by an increase in calcium influx, a decrease in calcium efflux, a release of calcium from intracellular pools, and/or a redistribution of calcium between intracellular compartments.

Lanthanum inhibits transmembrane calcium flux in several cell types, including myocardial cells, hepatocytes, and rat pancreatic acini (Korc and Schoni, 1987; Langer and Frank, 1972). As demonstrated in Figure 2A and B, ⁴⁵Ca²⁺ efflux and uptake were both inhibited (P < 0.05) by LaCl₃ in large cells. The observed increase in [Ca²⁺]ᵢ, therefore, was not due to a net increase in calcium influx into the large cell. However, inhibition of calcium efflux could effectively trap Ca²⁺ in the cytosol, if intracellular calcium stores were unable to effectively buffer changes in cytosolic calcium.

A change in [Ca²⁺]ᵢ was not observed in small luteal cells incubated with LaCl₃ (Figure 1C,D; Table 1), even though both calcium efflux and uptake were significantly inhibited (Figures 3A and 3B). No change in [Ca²⁺]ᵢ levels with LaCl₃ treatment was also reported in mouse lacrimal acinar cells (Kwan and Putney,
Since it was demonstrated that LaCl$_3$ inhibits transmembrane calcium fluxes in both large and small cells, the results provide evidence that the two cell types differ in their ability to regulate or buffer cytosolic [Ca$^{2+}$].

Because La$^{3+}$ displaces Ca$^{2+}$ from Ca$^{2+}$ binding sites, the possibility exists that the La$^{3+}$-induced apparent increase in [Ca$^{2+}$] in large cells was due to a direct interaction of La$^{3+}$ with FURA-2. Thus, a detailed series of control experiments were performed to eliminate this possibility. Fluorescence at 360 nm (isoexcitation point) is proportional to the total amount of intracellular FURA-2 and can be used to evaluate possible quenching effects. Lanthanum decreased the fluorescence at 360 nm in nominally Ca$^{2+}$-free medium, under cell-free conditions (Figure 4B). This suggests that La$^{3+}$ quenches the FURA-2 fluorescence. Binding of Ca$^{2+}$ to FURA-2 results in an increase in the 340/380 nm fluorescence ratio which can be transformed to changes in [Ca$^{2+}$], using equation [1] (Grynkiewcz et al., 1985). In a nominally Ca$^{2+}$-free, cell-free buffer, it was demonstrated that LaCl$_3$ alters FURA-2 (FA) fluorescence in a manner similar to the interaction of Ca$^{2+}$ with FURA-2 (i.e. an increase in the 340/380 nm ratio; Figure 3A, 3B). A similar effect of LaCl$_3$ on FURA-2 fluorescence was observed by Kwan and Putney (1990). Conversely, when FURA-2 was fully saturated with Ca$^{2+}$, addition of LaCl$_3$ did not affect FURA-2 (FA) fluorescence (Yamaguchi et al., 1987, unpublished).
data). This suggests that Ca$^{2+}$ competes more readily for FURA-2 than does La$^{3+}$. In summary, the above results demonstrate that La$^{3+}$ can directly interact with FURA-2 and alter the fluorescence at the three excitation wavelengths used for measurement of [Ca$^{2+}$].

Under conditions in which cellular viability is maintained, the action of La$^{3+}$ appears to be confined to the extracellular space (Kwan and Putney, 1990; Langer and Frank, 1972; Weiss, 1974; Wendt-Gallitelli and Isenberg, 1985). Within the time frame of the present experiments, cellular viability was maintained and La$^{3+}$ would not be expected to enter the cell. The following results suggest that the observed increase in 340/380 nm ratio with addition of LaCl$_3$ in large cells was due to a true increase in [Ca$^{2+}$], rather than a direct interaction of La$^{3+}$ with intracellular FURA-2. First, 360 nm fluorescence was not appreciably affected by addition of LaCl$_3$ in large or small cells (Ca$^{2+}$-replete medium, Figure 1B and 1D). Second, addition of the calcium ionophore A23187 (1 μM) at the end of the incubation period had no additional effect on 360 nm fluorescence, even though the 340/380 nm ratio was markedly increased. This result suggests that La$^{3+}$ does not have access to the intracellular compartment even under conditions of increased permeability to calcium. And third, unlike large cells, no change in 340/380 nm ratio was observed in small (Figure 1C and 1D, Table 1). It may be that large cells are more "leaky" to La$^{3+}$ than small. In "leaky" cells, La$^{3+}$ would
have a greater opportunity to bind to intracellular FURA-2. Membrane permeability to La$^{3+}$ was not measured in these studies, however, if La$^{3+}$ was "leaking" into large cells, it is unlikely that $^{45}\text{Ca}^{2+}$ uptake and efflux would have been so markedly inhibited by LaCl$_3$.

The La$^{3+}$-induced increase in $[\text{Ca}^{2+}]_i$ was inhibited in cells loaded with the intracellular calcium chelator, BAPTA (Figure 5). Due to the ability of LaCl$_3$ to displace calcium, the possibility exists that BAPTA also chelates La$^{3+}$. If this were the case, cytosolic-free La$^{3+}$ would compete with cytosolic-free Ca$^{2+}$ for binding to BAPTA and more $[\text{Ca}^{2+}]_i$ would be available to and produce an increase in the 340/380 fluorescence ratio of FURA-2. The ability of BAPTA to block the La$^{3+}$-induced increase in fluorescence suggests that the increase in 340/380 nm fluorescence was due to a true increase in $[\text{Ca}^{2+}]_i$. Taken together, all of the above results support that the increase in 340/380 nm ratio in large cells is due to an increase in $[\text{Ca}^{2+}]_i$.

The Kd$_a$ of FURA-2 exhibits a sensitivity to pH (Ganz et al., 1990; Martínez-Zaguilán et al., 1991). Thus, a La$^{3+}$-induced change in pH$^{\text{in}}$ might cause a change in the 340/380 nm ratio independent of changes in $[\text{Ca}^{2+}]_i$ due to a change in the Kd$_a$. No change in pH$^{\text{in}}$ was observed with addition of La$^{3+}$ in large or small cells (Figure 6), therefore, this was not the case.
Continuous leakage of FURA-2 has been previously demonstrated in untreated large and small luteal cells. Furthermore, this leakage could be blocked by the anion transport inhibitor, probenecid (McDonough and Burton, 1989). If FURA-2 was leaking from the cell, then the gradual increase in 340/380 nm ratio could be due to the interaction of La\(^{3+}\) with extracellular FURA-2. However, in large cells incubated with probenecid, the La\(^{3+}\)-induced increase in \([\text{Ca}^{2+}]_i\) was still observed, and the increase was similar to that in large cells incubated in the absence of probenecid (Figure 7). The rate of dye leakage from small cells was similar to large cells (Figure 1A and 1C), but a change in the 340/380 nm ratio was not observed in small cells. Taken together, these observations suggest that in large cells, the increase in 340/380 ratio in response to LaCl\(_3\) is not due to a direct interaction of La\(^{3+}\) with FURA-2 that has leaked from the cells. Finally, a quenching effect of La\(^{3+}\) on FURA-2 fluorescence was observed in this study. However, evidence from a series of control experiments strongly suggests that the increase in the 340/380 nm ratio observed only in large cells, was due to a true increase in \([\text{Ca}^{2+}]_i\).

In summary, the results of this study provide evidence that large, as compared with small luteal cells, have a reduced ability to regulate or buffer \([\text{Ca}^{2+}]_i\) by intracellular mechanisms. A dependence on calcium efflux as a primary mechanism to handle a calcium load, a reduced concentration of intracellular
calcium buffers, or a reduced ability to sequester cytosolic-free calcium into intracellular calcium compartments in large as compared with small cells could explain the differences in \([\text{Ca}^{2+}]_i\) response between the two cell types. Such a reduced ability may predispose large cells to cytotoxic effects of sustained increases in \([\text{Ca}^{2+}]_i\), known to be induced in these cells by PGF$_{2\alpha}$. The reduced ability to regulate \([\text{Ca}^{2+}]_i\) may therefore relate to the apparent specificity with which large cells respond to a PGF$_{2\alpha}$-induced signal for luteal regression. To further test this possibility, future studies will investigate differences between large and small cells in mechanisms that regulate Ca$^{2+}$-efflux and in the calcium uptake capacity of various intracellular organelles known to sequester calcium.

ACKNOWLEDGEMENTS.

The authors wish to thank Mr. S.L. Marion, Mr. W. Engleberg, and Ms. G.M. Martinez for technical assistance. This work was supported by HD20613, BRSG # 2507, RR05675, GM40337, HL07249.
REFERENCES


ABBREVIATIONS USED

\( \text{pH}^\text{in} \), intracellular pH; \( \text{pH}^\text{cyt} \), cytoplasmic pH; \( \text{pH}^\text{ves} \), endosomal/lysosomal pH; 
\( \text{pH}^\text{ex} \), extracellular pH; \( [\text{Ca}^{2+}]^\text{in} \), intracellular calcium; \( R \), ratio; \( R_{\text{max}} \), maximum ratio; 
\( R_{\text{min}} \), minimum ratio; \text{CHO cells}, Chinese Hamster Ovary cells; \text{EAT cells}, Ehrlich ascites tumor cells; \text{NEM}, N-ethylmaleimide \([1\text{-ethyl-1H-pyrrole-2,5-dione}]\); 
\text{Suramin}, \([\text{Hexasodium sym-bis}(m\text{-aminobenzyl-}m\text{-amino-}p\text{-methylbenzoyl-1-naphtylamino-4,6,8-trisulfonate})\text{carbamide}]\); \text{Fusidic acid}, \([16\text{-}(\text{Acetyloxy})\text{-3,11-dihydroxy-29-dammar-a-17}(20),\text{24-dien-21-oic acid}]\); \text{SCH 28080}, \([2\text{-methyl-8-(phenylmethoxy)}\text{-imidazole}]\); \text{DMSO}, dimethyl sulfoxide; \text{Pyranine}, 8-hydroxypyrene-1,3,6-trisulfonic acid; \text{BCECF}, 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein; \text{SNARF-1}, CarboxySeminaphtorhodafluor; \text{FURA-2}, 5-oxazolecarboxylic acid, 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-bis(carboxymethyl)amino)-5-methylphenoxy-2-benzofuranyl); \text{BAPTA}, bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; \text{X-AM}, acetoxymethylester form of fluoroprobe X; \text{HEPES}, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; \text{MES}, 2-(N-morpholino)ethanesulfonic acid; \text{MOPS} = 3\text{-}(N\text{-morpholino})propanesulfonic acid; \text{EGTA}, ethylene glycol-bis(b-aminoethyl ether) -N,N,N',N'-tetraacetic acid.