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SMITH, PETER FRANCIS

THE PHARMACOLOGICAL CHARACTERIZATION OF 2,4-DIAMINO-5-CYANO-6-BROMOPYRIDINE (COMPOUND I), A NON-XANTHINE PHOSPHODIESTERASE INHIBITOR, AS A BRONCHODILATOR IN THE RABBIT

THE UNIVERSITY OF ARIZONA

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A NON-XANTHINE PHOSPHODIESTERASE INHIBITOR,
AS A BRONCHODILATOR IN THE RABBIT

by

Peter Francis Smith

A Thesis Submitted to the Faculty of the
TOXICOLOGY PROGRAM
DEPARTMENT OF PHARMACOLOGY and TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
WITH A MAJOR IN TOXICOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1983
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This thesis has been approved on the date shown below:

David G. Johnson, M.D.
Professor and Head
Section of Endocrinology
Department of Internal Medicine

Date
DEDICATION

This thesis is dedicated to my wife, Sandra, and my son, Peter, for their moral support and love but most of all for their bearing with me during my attempts to satisfy my curiosity.
ACKNOWLEDGEMENTS

This author's graduate career has truly been a positive and enlightening learning experience, the greatest part of this experience being contributed by the associations made with fellow researchers and educators. The lesson obtained during this period of my life which is most outstanding in my mind is that achievements in research are not based solely on individual efforts but rather occur as the result of a cooperative effort on the part of an established research community; a community in which intellectual freedom and creative thinking are encouraged and, in a less philosophical vein, a community where people share ideas, equipment, and above all a common curiosity to identify the processes by which things "tick." To these people, all of whom are members of the research community in which I was trained, I sincerely thank you for making me believe that it all may have been worth the trip; Doctors David G. Johnson, Marilyn Halonen, John D. Palmer, Robert Volp, W. Joseph Thompson, I. Glenn Sipes, Dean Carter, Klaus Brendel, and Murray Korc. Also to I. Carla Lohman and Anita Farland.

Finally, I wish to thank Mrs. Delores Romero for her time and effort in the preparation of this manuscript.
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ABSTRACT

2,4-Diamino-5-cyano-6-bromopyridine (compound I) was previously shown to inhibit cyclic AMP-phosphodiesterase in vitro in both dog kidney and rat pancreatic islet tissue. This suggested that it might have smooth muscle relaxant, hence bronchodilatory, properties similar to other known cyclic AMP-phosphodiesterase inhibitors such as methyl isobutylxanthine (MIX). Compound I or MIX significantly reduced the bronchoconstriction produced by 2 μM carbachol in a similar fashion. Histamine-induced constriction of rabbit bronchi was also inhibited by similar concentrations of compound I, and antigen-induced bronchoconstriction of sensitized bronchi was similarly attenuated by compound I pre-treatment. Finally compound I was shown to inhibit the cyclic AMP phosphodiesterases (low K_m) of rabbit bronchial smooth muscle and human peripheral lung. These findings suggest that compound I is a potent bronchodilator which acts via bronchial cyclic AMP phosphodiesterase inhibition.
CHAPTER 1

INTRODUCTION

Bronchial Asthma: Therapy Through the Ages

Bronchial asthma has been characterized as the end result of an extremely complex series of indirectly related pathophysiological events, the clinical manifestations of which have been known since antiquity (Rosenblatt, 1976). Reports on the existence and descriptions of this disease have been traced back as far as the Hippocratic writings (460-370 B.C.) (Peterson, 1946), with the earliest known description recorded sometime during the early Christian era (2nd century) by Aretaeus, in which the patient was described in much detail as expressing facial anxiety, rapid respirations, scant, foamy expectoration, and fear of suffocation (Adams, 1856). Explanations regarding the cause included the production of dampness of the breath by a viscid humor in the lungs, the primary site of the disease.

Galen (131-201 A.D.) regarded asthma as a mechanical paroxysmal interruption of respiration caused by the accumulation of superfluous, thick secretions coming down from the brain (Daremberg, 1854). The 7th century physician, Paulus Aegineta, attributed the origin of the disease to
"thick and viscid humors becoming infarcted in the bronchial cell of the lung." He believed that there were no structural changes in the lung in ordinary convulsive asthma, but that changes might occur as a result of engorgement of the lining membranes of the bronchi. Aegineta's therapeutic approach included vinegar of squills, cow parsnip, mustard, and round birthwort as well as external applications of figs, honey, wax, flours of iris, and barley. Supplementary procedures consisted of bleeding, purging, and inducing emesis (Adams, 1844).

The emergence during the Renaissance of independent thought in the medical sciences resulted in a disregard for many of the earlier etiological interpretations of asthma. Joannes Van Helmont, in refuting the Galenic concept of secretions coming down from the brain, held that asthma originated in the "pipes of the lungs." His most important contribution was his association of asthma with seasonal changes, inhaled irritants, and ingestion of particular foods (Van Helmont, 1662). The prominent 17th century physician, Daniel Sennert, believed that a viscid material which originated in the liver was carried by the circulation to collect in and obstruct the trachea and bronchi and to subsequently cause asthma (Sennert, 1641). Therapy by Sennert's contemporary, Lazare Riviere, included phlebotomy, clysters, emetics, and expectorants (Riviere, 1640). Thomas Sydenham, the "English Hippocrates" conceived of asthma as
being a symptom of an underlying disease in which the "bronchi are stuffed up." He followed traditional therapy emphasizing general measures such as bleeding and purging on a regular schedule (Latham, 1850). In 1769, John Millar was the first to consider asthma as a definitive clinical disease with its own mode of onset, clinical course, and subsequent complications (Millar, 1769), and Fitzgerald, in 1802, was the first to implicate heredity as one of the predisposing factors, thereby suggesting atopic genetic involvement.

Laennec's classic publication on auscultation in 1819 and his invention of the stethoscope considerably advanced the state of knowledge of pulmonary diseases. He defined asthma as a paroxysmal dyspnea occurring between two intervals of normal respiration and he attributed the physiological basis for the attacks to spasmodic contraction of the muscle fibers around the bronchi and air vesicles. His therapy included squills, ipecac, narcotics, dilute prussic acid, emetics, alkaline salts, and volatilized oxides of lead (Laennec, 1834).

In 1864, Dr. Hyde Salter published a monograph on asthma. He observed that asthma-like symptoms occurred in cases of hay fever and were provoked in certain individuals by contact with rabbits or cats. His therapy included expectorants, emetics, and the smoking of stramonium tobacco
as well as the inhalation of chloroform (Salter, 1864). Several years earlier (1859), Salter had published a report in the Edinburgh Medical Journal in which he recommended two large cups of strong coffee as the best remedy for asthma (Salter, 1859). We know today that what Salter was effectively prescribing in the mid-1800's was anti-cholinergic (stramonium leaves) and anti-phosphodiesterase (caffeine) therapy, the latter of which is a common mode of therapy today (i.e., phosphodiesterase inhibitors).

The first experimental observation of anaphylaxis was published by Megendie (1839) in his Lectures on the Blood. He demonstrated that subsequent injections of egg albumin produced death in rabbits tolerant to the initial injection. In 1902, Richet showed a similar phenomena using extracts of actinia in which a toxic reaction occurred and he termed this reaction "anaphylaxis" for loss of protection (Richet et al., 1902).

In the 20th century, we have experienced a tremendous acceleration of accumulated biomedical knowledge with regard to the etiological basis of bronchial asthma. As witness to this knowledge a recent definition of asthma describes the disease as follows:

...a disease characterized by wide variations over short periods of time in resistance to flow in the airways of the lungs. Increases in resistance to air flow may be related to exposures to environmental factors, especially inhaled substances in concentrations that do not affect the majority of
persons, or they may occur without apparent external cause. Detectable factors include specific antigen-antibody reactions, usually to inhaled antigens; hyperreactivity of the airways to a variety of physical and chemical stimuli; and exercise.

(J.G. Scadding, 1976)

Such "mechanistic" determinations regarding etiology have allowed the science of drug design to take a more rational approach as these specific pathophysiological alterations become "specifically - targeted" for therapeutic drug interactions.

In light of this advancing knowledge, corresponding advances in pharmacological research which involve the treatment of asthma have led to major improvements in drug therapy. Currently, one of the single most important concepts which has emerged relative to the drug treatment of asthma (Szentivanyi, 1968) involves the role of cyclic nucleotides; specifically cyclic adenosine monophosphate (cyclic AMP), which has been shown to effectively block the release of chemical mediators responsible for the onset of anaphylaxis (Kaliner and Austen, 1974, and Orange et al., 1971 a & b) as well as to cause relaxation of bronchial smooth muscle, hence bronchodilatation (Schultz et al., 1972).

Drugs which have been shown to act via mechanisms which ultimately increase cellular levels of cyclic AMP have proven invaluable in the treatment of asthma and these include two fairly broad categories; those drugs which are
effective β-adrenergic agonists (β-adrenergic hormones, drugs such as isoproterenol and salbutamol, prostaglandins, cholera toxin, etc.) which directly turn on the synthesis of cyclic AMP via the adenylate cyclase enzyme-receptor complex (Robison et al., 1971), and those drugs which influence cyclic AMP levels through a post-receptor event mechanism, namely inhibition of cyclic AMP-phosphodiesterase, the enzyme responsible for inactivating cyclic AMP by hydrolysing it to 5'-adenosine monophosphate (Robison et al., 1971).

This second category of drugs, which indirectly increase levels of cyclic AMP, includes the methylxanthines such as theophylline, caffeine (Butcher et al., 1968), and 1-methyl-3-isobutylxanthine (Beavo et al., 1970), the bischromone disodium cromoglycate (Rachelefsky et al., 1975, and Taylor et al., 1974a), and, among others, a recently introduced substituted halo-pyridine with potential anti-asthmatic activity, 2,4-diamino-5-cyano-6-bromopyridine (compound I) which is the major focus of this paper.

Compound I (figure 1) is a substituted bromopyridine which was originally synthesized in 1957 (Carboni et al., 1958). The compound is a yellowish-white powder with a melting point between 260 °C and 265 °C and a formula weight of 214. Interest in compound I's potential pharmacological properties first appeared in the literature in 1976 when
2,4-Diamino-5-Cyano-6-Bromopyridine (Comp. 1)
Hershfield and Richards investigated a series of pyridine derivatives on the basis of structural similarity to glucose and to the hydrogen bonding capacity of this sugar. These compounds, which included compound I, were found to reversibly inhibit glucose transport in a systematic fashion in the human erythrocyte (Hershfield and Richards, 1976).

Based on these findings, Johnson and De Haen (1979) investigated the consequences of various 2,4-diamino-5-cyano-6-halopyridines on insulin secretion in the perfused rat pancreas; glucose being the primary physiological regulator of insulin secretion in most mammals. In these experiments, compound I was found to increase insulin release 7 to 20-fold above that caused by glucose alone. However, this response required elevated glucose concentrations which is similarly a requirement for insulin secretagogues whose effects on the pancreatic β-cell are mediated by increased levels of cyclic AMP (Malaisse et al., 1967, and Johnson et al., 1973). Therefore, the possibility that compound I was acting via cyclic AMP-phosphodiesterase inhibition was considered and subsequently investigated. Compound I was shown to significantly inhibit cyclic AMP-phosphodiesterase in vitro using either purified dog kidney enzyme or homogenates of rat pancreatic islet tissue (Johnson et al., unpublished observation).
The fact that compound I inhibited cyclic AMP-phosphodiesterase in kidney and pancreas suggested that it might also inhibit this enzyme in bronchial smooth muscle with the anticipated result of smooth muscle relaxation. This possibility was investigated by Smith and Johnson (1983) who demonstrated that compound I did indeed inhibit the cyclic AMP-phosphodiesterase(s) of both rabbit bronchial smooth muscle and human lung tissue in addition to causing significant relaxation of pre-constricted bronchial smooth muscle using an in vitro preparation of suspended bronchiolar rings from the rabbit. Compound I was also shown to attenuate the constrictor response of bronchiolar rings to histamine, and to reduce the degree of antigen-induced constriction in sensitized bronchial smooth muscle (Smith et al., 1983).

The Role of Cyclic AMP in Asthma:
I) Cyclic AMP and Mediator Release

The role of cyclic AMP in asthma is indeed paramount in importance when one considers the possibility that, as a single, common mediator, it has the potential to block the release of the chemical mediators of anaphylaxis in addition to reversing the bronchoconstriction produced by these mediators.

Type I hypersensitivity reactions (anaphylaxis) have been shown to result from the binding of a homocytotropic antibody (IgE) via its $F_c$ fragment to specific
receptor sites on the surfaces of mast cells or basophils. Antigen reacts with the cell-bound antibody to form biva-
lent complexes which in turn trigger a series of enzymatic
reactions leading ultimately to the release of chemical
mediators which include histamine, serotonin (5-hydro-
xytryptamine), slow-reacting-substance of anaphylaxis
(SRS-A), and eosinophil chemotactic factor of anaphylaxis,
ECF-A (Ishizaka et al., 1966, and Johansson and Bennich,
1967).

Upon release of chemical mediators a variety of
physiological alterations occur; among the most important
are increased vascular permeability and bronchiolar smooth
muscle contraction which leads to bronchoconstriction,
edema, and increased mucus formation characteristic of
asthma.

The cells which release these mediators possess
cytoplasmic granules which "degranulate" following the
interaction of antigen with IgE (and possibly IgG) anti-
body. These cells are thought to play a role in defense
against foreignness. However, when over-stimulated in the
atopic individual, they lead to the production of clinical
symptoms via the release of vasoactive amines. Most of the
IgE fixed in the lung is present on the mast cells which
are the principle site of challenge and are regarded as the
major source of bronchoreactive agents. These cells are
situated in close proximity to the bronchial smooth muscle,
vascular system, and glands of the airways which, by virtue of this association, are passive recipients of the agents released from the mast cells (Bellanti, 1971).

Studies have indicated that the process of mediator release is modulated by the intracellular levels of cyclic AMP. In these investigations, researchers employed human peripheral leukocytes (Lichtenstein and Margolis, 1968) and lung (Orange et al., 1971b, and Tauber et al., 1973) and nasal polyp fragments (Kaliner et al., 1973). Results have demonstrated an inverse relationship between increased cellular levels of cyclic AMP after stimulation with beta-adrenergic agents, prostaglandins, cholera toxin, and methylxanthines, and the capacity of antigen challenge to induce the release of mediators. It has also been shown that the depletion of intracellular cyclic AMP after treatment of lung tissue with imidazole (Kaliner and Austen, 1974b), low concentrations of prostaglandins (Tauber et al., 1973), or alpha-adrenergic stimulants (Kaliner et al., 1972), is associated with enhancement of IgE-mediated histamine and SRS-A release. Therefore, a reciprocal relationship exists between the intracellular levels of cyclic AMP and the capacity for the union of antigen and IgE to induce the release of mediators from mediator cells.
The mechanism of IgE-dependent mediator release in human atopic basophils was characterized by Hastie (1971) to be a non-cytotoxic event which is essentially a form of immunologically induced secretion.

This process has been partially elucidated by Kaliner and Austen (1973) who describe the series of events following the union of antigen with IgE as follows . . . a) the initiation of a calcium-dependent activation of an enzyme precursor to a diisopropyl-fluorophosphate (DFP) sensitive serine esterase; b) a further autocatalytic feedback activation of the proesterase; c) an energy-requiring step which utilizes either glycolysis or oxidative phosphorylation; d) an intracellular step requiring calcium (and inhibited by EDTA); e) a stage which is suppressed by increased concentrations of cyclic AMP, and finally; f) mediator release. (see figure 2).

The specific mechanism by which cyclic AMP prevents mediator release has not been determined. However, it has been suggested, based on studies by Goodman et al. (1970), and Lichtenstein and Henney (1972), that a cyclic AMP-dependent protein kinase may inhibit the release reaction by phosphorylation of microtubular proteins. These microtubules are thought to be responsible for moving the mediator-filled storage granules to the plasma membrane where the storage granule membrane fuses with the plasma-
Figure 2. The pharmacological control of the immunological release of chemical mediators from human lung tissue. Note the role of cyclic AMP in this process and the influence which cyclic AMP-phosphodiesterase inhibitors (methylxanthines and possibly compound I) have on this process. Adapted from Kaliner, M., and Austen, K.F. Cyclic nucleotides and modulation of effector systems of inflammation. Biochem. Pharm. 23:763, 1974.)
membrane resulting in a "pore" which allows for the escape of the contents of the granules or the entrance of extracellular ions. The histamine is held loosely in a complex with protein and highly charged acidic polysaccharide from which it is quantitatively replaced by sodium. The fused granules simply lose their histamine. Upon reestablishment of the membrane, histamine is resynthesized by the mast cell while the sodium is removed by the membrane pump and the storage granules are restocked (Brocklehurst, 1976).

Reduction of phosphorylation by depletion of cyclic AMP levels or activation of a cyclic GMP-dependent phosphatase may enhance mediator release. A number of pharmacological agents which would be expected to increase intracellular levels of cyclic AMP have been shown to inhibit mediator cell secretion (Assem and Richter, 1971, Lichtenstein and Margolis, 1968, Perper et al., 1972, and Lichtenstein et al., 1973) and, conversely, agents which should decrease cyclic AMP levels, enhance secretion (Kaliner et al., 1972, and Orange and Austen, 1971).

To reiterate, it is quite clear that cyclic AMP holds a most important position in terms of the biochemical processes controlling mediator release during anaphylaxis. The mechanism of action of beta-adrenergic agents and phosphodiesterase inhibitors is only speculative. While it is generally believed that they act directly on smooth muscle,
it seems as likely that they exert their effect by decreasing the rate of mediator release. In any case, it is obvious that there exists a great therapeutic potential in the treatment of asthma for agents which act to alter the concentration of this most important cyclic nucleotide.

II) The Role of Cyclic AMP in Bronchodilatation

Clearly, cyclic AMP has been established as an important modulator of the processes involving the release of chemical mediators during anaphylaxis. However, in recent times, its role in these processes has been somewhat overshadowed by its hypothetical control of bronchial smooth muscle relaxation; the response attributed to those chemicals which are known $\beta_2$-adrenergic receptor agonists and (or) cyclic AMP-phosphodiesterase inhibitors and which effectively increase intracellular levels of cyclic AMP.

There is much current debate over the question of mechanism of action with regard to bronchodilators. In light of the discovery by Sutherland and Robison (1966) that $\beta$-adrenoceptor stimulants increase cyclic AMP by stimulating adenylate cyclase, coupled with the findings that either $\beta_2$-adrenergic agonists (Ahlquist, 1948; Lands, 1967; Andersson, 1972; Jack, 1973; and Moran, 1975) or cyclic AMP-phosphodiesterase inhibitors (Amer and Kriehbaum, 1975; Polson et al., 1978; Triner et al., 1977; and Newman et al., 1977) could be used therapeutically to produce
bronchodilatation, it has generally been assumed that cyclic AMP is the mediator of this response (Somlyo and Somlyo, 1970; Andersson, 1972; and Bar, 1974).

In support of this concept the following observations have been made:

- several groups have shown that, for a number of chemically divergent agents, there is a statistically significant correlation between the in vitro phosphodiesterase inhibiting activity and the capacity to produce bronchodilatation (Triner et al., 1977; Newman et al., 1977, Polson et al., 1978; and Smith et al., 1983).

- substantial evidence has accumulated for cyclic AMP playing a role in the relaxing actions of β-adrenergic and some other agents. For instance, β₂-adrenergic and Prostaglandin stimulation cause an increase in the intracellular concentration of cyclic AMP and a relaxation of most smooth muscle tissues (Bar, 1974), and,

- the dibutyryl derivative of cyclic AMP, a highly lipid-soluble thus membrane-penetrable derivative, when exogenously applied to various smooth muscle preparations has been shown to cause relaxation in all preparations studied (Bar, 1974) and to mimic the hyperpolarizing effect of β-adrenergic stimulation in vascular smooth muscle (Somlyo et al. 1972).

Conversely, the following studies appear to deny the role of cyclic AMP as the mediator of bronchial smooth muscle relaxation;

- accumulating data indicates that theophylline, the most widely used therapeutic phosphodiesterase inhibitor, at therapeutic concentrations, is only a weak inhibitor of phosphodiesterase activity in vivo yet bronchodilatation is pronounced following administration of this drug. As a
result, there is reason to suspect that inhibition of the phosphodiesterase enzyme system, hence increased cyclic AMP levels, is not the sole mechanism of bronchodilatation produced by this drug and other phosphodiesterase inhibiting bronchodilators. In studies with theophylline, Lohnmann et al. (1977) reported that at concentrations of 10 μM, theophylline relaxed carbachol-contracted bovine tracheal muscle by 85-95% but did not significantly elevate control levels of cyclic AMP. A similar conclusion was reached by Kolbeck et al. (1979) using either guinea-pig tracheal rings or tracheal muscle strips from the dog.

- in the intact lung, there was no demonstrable potentiation of bronchodilatation by drugs which increase cyclic AMP. More specifically, caffeine (a phosphodiesterase inhibitor) did not potentiate the bronchodilator action of epinephrine in the dog, even though both drugs acted synergistically in increasing the levels of cyclic AMP in rat lung slices (Oskoui et al., 1970), and,

- in a variety of smooth muscle preparations, cyclic AMP has been applied exogenously in attempts to mimic the effects of hormones or other agents that raise cyclic AMP levels and cause relaxation. Cyclic AMP caused relaxation only in some preparations, whereas it promoted contraction or augmentation of contractile responses to other agents in some other smooth muscle tissues (Bar, 1974).

In view of the conflicting reports concerning the mechanism of action of bronchodilators and the role of cyclic AMP in this process, it is evident that a clear and defensible elucidation awaits further investigation. However, some recent work in the field of smooth muscle biochemistry has resulted in a theory that conceivably provides a direct role for cyclic AMP in smooth muscle relaxation (Adelstein, 1978). The mechanism upon which this theory is based would conveniently accommodate the cyclic
AMP-mediated theory of bronchodilatation which has been extended over the past 15 years. It would also allow for agreement with previously suggested mechanisms which purport to explain the production of bronchoconstriction in bronchial asthma, such as the opposing effects of cholinergic and β-adrenergic stimuli in modulating bronchomotor tone which has been well demonstrated in vivo (Cabézas, 1971), enhanced cholinergic and β-adrenergic mechanisms (Simonsson, 1972), and diminished β-adrenergic mechanisms (Szentivanyi, 1968). All of these mechanisms appear to exert their effects through a common biochemical pathway; by altering cyclic nucleotide metabolism.

The initial finding which prompted this most timely theory was that the cyclic AMP-dependent phosphorylation of turkey gizzard smooth muscle myosin kinase resulted in a 2-fold decrease in the rate at which the enzyme phosphorylated the light chain of smooth muscle myosin, which regulates actin-myosin interaction in smooth muscle. These results suggest that cyclic AMP has a direct effect on actin-myosin interaction in smooth muscle and that this interaction may lead to smooth muscle relaxation.

In smooth muscle, cyclic AMP derivatives or agents which increase intracellular levels of cyclic AMP (i.e., phosphodiesterase inhibitors), inhibit tension development. This has largely been attributed to enhanced binding of
Ca\(^{2+}\). Adelstein et al. (1978) suggest a mechanism by which decreased cyclic AMP levels can directly affect smooth muscle contraction. A rise in calcium concentration results in a decreased level of cyclic AMP by activating phosphodiesterase activity. Decreased levels of cyclic AMP lead to an increase in the dephosphorylated (more active) form of the myosin kinase, amplifying the effect of calcium, which is required for myosin kinase activation. The end result is an increase in phosphorylated myosin which, unlike unphosphorylated myosin, can interact with actin. In this sequence cyclic AMP, by activating a cyclic AMP-dependent protein kinase that can phosphorylate myosin light chain kinase, causes a decrease in the activity of the enzyme thus favoring the unphosphorylated form of myosin which cannot interact with actin to produce smooth muscle contraction (figure 3).

Therefore, drugs which act to increase intracellular levels of cyclic AMP could, by virtue of this proposed mechanism, produce smooth muscle relaxation.

**Phosphodiesterase Inhibitors in Asthma**

Based on the information which has been presented thus far, detailing the primary importance of cyclic nucleotide involvement in asthma, it should be obvious that drugs which influence the balance of cyclic nucleotides
Figure 3. Schematic representation of one mechanism by which cyclic AMP and calcium have been proposed to regulate smooth muscle contraction. Note that the net effect of the cyclic AMP sequence is to inhibit the phosphorylation of myosin, thus favoring relaxation. Adapted from Adelstein R., and Hathaway, D. Role of Calcium and Cyclic Adenosine 3':5' monophosphate in regulating smooth muscle contraction. Am. J. Card. 44; 73, 1979.
might also alter the outcome, or perhaps prevent the occurrence of an asthmatic attack. In particular, drugs which inhibit the cyclic AMP-phosphodiesterase(s), the only known pathway for the metabolism of cyclic AMP (Beavo et al. 1970), would be expected to yield an increase in the intracellular levels of cyclic AMP by preventing the hydrolysis of this nucleotide. This elevation of cyclic AMP in response to phosphodiesterase inhibition has been widely observed (Beavo et al. 1970, Butcher et al. 1968, and Helfman and Kuo, 1982), and it is generally assumed that drugs which inhibit phosphodiesterases exert their physiological effects secondary to increasing intracellular levels of cyclic AMP.

Phosphodiesterase inhibitors used in the control of asthma have a long history beginning with the methylxanthines which were the first phosphodiesterase inhibitors to be used for the treatment of this disease. Caffeine was probably the first recorded methylxanthine to be employed for this purpose and its use dates back to the mid-1800's when Salter (1859) prescribed two large breakfast cups of strong coffee as the best remedy for asthma. On an empty stomach, this should be a bronchodilating dose of caffeine.

In 1890, Parisot demonstrated that caffeine prevented exercise-induced asthma, and Pal (1912), and Trendelenburg (1912) observed that caffeine was a bronchodilator in vitro and in vivo in animals. In 1922,
Hirsch observed that a mixture of methylxanthines (theophylline and theobromine) produced marked bronchodilatation in asthmatics, and Herrmann et al. (1937) showed that theophylline could successfully relieve 'status asthmaticus'. Subsequent to these findings, theophylline gained widespread popularity as a major drug used for the relief of asthma. Today it is one of the most popular agents for the treatment of this disease, both acutely and prophylactically.

Theophylline has been shown to successfully relax bronchial smooth muscle (Newman et al. 1977), and Persson and Eckman, 1976), relieve pulmonary hypertension (Werko and Lagerlof, 1950, and Parker et al. 1966), stimulate mucociliary clearance (Serafini et al. 1976, and Matthys, 1980), inhibit the release of anaphylactic mediators (Lichtenstein and Margolis, 1968), and suppress mediator induced edema in the lung (Persson et al. 1979). Experimentally, theophylline is used as an inhibitor of phosphodiesterase for investigational purposes (Bergstrand, 1980), and as a specific antagonist to adenosine actions (Fredholm, 1980).

However, one of the problems associated with theophylline therapy is the drug's narrow therapeutic index. Serious side effects have been associated with theophylline, including convulsive and arrhythmogenic
actions (Andersson and Persson, 1980). It is also particularly unfortunate that the elimination rates of theophylline vary greatly between individuals (Jenne et al. 1976).

Since the finding that the methylxanthines inhibited phosphodiesterase (Butcher and Sutherland 1962), and that this inhibition might explain their mode of action, there has been a major effort to produce pharmacological agents that would selectively inhibit cyclic nucleotide phosphodiesterases. The rationale pursued in these investigations was to find drugs that would act (a) by competing with the cyclic nucleotide substrate; (b) by acting at the substrate site non-competitively; (c) by acting at an allosteric site to increase or decrease the affinity of the enzyme for its substrate; and (d) by acting on one of the endogenous activators or co-factors for the enzyme. The methylxanthines, which have a structural similarity to the cyclic nucleotides, competitively inhibit phosphodiesterase (Butcher and Sutherland, 1962).

Part of the problem encountered in developing a selective phosphodiesterase inhibitor has to do with the ubiquitous nature of these enzyme systems and their substrates (cyclic AMP and cyclic GMP) which are found in virtually all cell types (Amer and McKinney, 1973) with the possible exception of red blood cells (Drummond and
Perrott-Yee, 1961) and isolated rat adrenal cells (Kitabchi et al., 1971). Therefore, it would seemingly be difficult to exert control over a specific target tissue. However, this problem may be overcome because the metabolism of the cyclic nucleotides is controlled by an extremely complex system of enzymes consisting of a variety of isozymes and receptor subunits (Applemann et al. 1973). Moreover, the characteristics of these enzyme systems differ between tissues and even between cell types (Uzunov et al. 1974). Hence, the development of pharmacological agents which could take advantage of these characteristic differences in the enzymes might result in the selective alteration of cyclic nucleotide metabolism only in the diseased or targeted tissue. In this regard, research into selective phosphodiesterase inhibition is justified.

Besides theophylline, it appears that, of the multitude of compounds found to inhibit phosphodiesterase(s) (Amer and Kreighbaum, 1975), only a small number have shown any success in the treatment of experimental and (or) clinical asthma, although a substantially larger number have been shown to cause smooth muscle relaxation. Of those agents shown to be effective in the treatment of experimental asthma, Davies (1973) demonstrated the effective protection against histamine challenge in conscious guinea-pigs with the triazolopyrazine, ICI 58 301, and the
triazolopyrimidine, ICI 63 197. These two compounds have been described as potent bronchodilators as well as phosphodiesterase inhibitors, and they have been shown to have no central nervous system or cardiovascular side effects, a downfall of the methylxanthines. Two other agents, doxantrazole and carboxythioxanthone, were shown to be potent phosphodiesterase inhibitors as well as to possess the capability to prevent histamine release from antigen-challenged guinea-pig lungs in vitro (Tateson and Trist, 1975). However, the bronchodilator potential of these compounds was apparently not investigated.

Most recently, Smith et al. (1983) have demonstrated the halogenated pyridine, 2,4-diamino-5-cyano-6-bromopyridine (compound I), to be a potent phosphodiesterase inhibitor as well as a bronchodilator in vitro in the rabbit. Preliminary studies indicate that this compound may also attenuate systemic anaphylaxis in the anesthetized rabbit.

Clinically proven antiasthmatics other than theophylline which may act by cyclic nucleotide phosphodiesterase inhibition include disodium cromoglycate and certain steroids. Sodium cromoglycate is an agent that blocks the release of chemical mediators from bronchial mast cells following the union of antigen with antibody (Taylor et al., 1974b). The drug is taken by inhalation and is used
to prevent the occurrence of asthma caused by exposure to allergens rather than to treat bronchospasm. This drug may act through cyclic AMP-phosphodiesterase inhibition, although it has been described as a weak inhibitor (Roy and Warren, 1974). For example, lymphocytes of patients receiving disodium cromoglycate do, in fact, have lower activities of phosphodiesterase than do those of untreated or theophylline-treated patients (Lavin et al. 1975a).

Finally, certain steroids used prophylactically in treating asthma may also act by altering the metabolism or action of cyclic nucleotides. This is predicted on the basis that glucocorticoids augment many physiological responses mediated by cyclic AMP, such as lipolysis and glycogenolysis (Brodie et al. 1966). In this regard, hydrocortisone (Lavin et al. 1975b) and dexamethasone (Manganiello and Vaughan, 1972) have been reported to inhibit phosphodiesterase. However, they are relatively weak inhibitors and therefore may act instead via an alternative mechanism (i.e., by increasing adenylate cyclase activity, or by increasing the synthesis of a cyclic AMP-dependent protein kinase).

Many of the drugs which are currently used to treat asthma have associated side effects which warrant careful dosing schedules and periodic monitoring of plasma levels. Although the drug combinations used to treat asthma are
quite effective, this limitation may present a problem. The ideal drug to treat asthma, that is one which is cheap, well absorbed orally, long acting, and has no systemic toxicity in addition to having a high therapeutic index while effectively suppressing or preventing the symptoms of asthma, eludes medical science. By virtue of the highly significant role which cyclic nucleotides have been shown to play in asthma, it is not unlikely that the search for such a drug may end with the discovery of a selective cyclic nucleotide phosphodiesterase inhibitor.

**Intent of Study**

2,4-Diamino-5-cyano-6-bromopyridine (compound I) has been shown to inhibit the cyclic nucleotide phosphodiesterase enzymes present in purified dog kidney preparations as well as in homogenates of rat pancreatic islet tissue. Based on these findings we decided to investigate the bronchodilatory potential of compound I due to the possibility that it might similarly inhibit the cyclic AMP-phosphodiesterases present in bronchial smooth muscle, resulting in smooth muscle relaxation.

In these studies compound I was characterized pharmacologically in terms of its smooth muscle relaxant effects using an *in vitro* technique of isolated bronchial rings from California rabbits. The effects of compound I were compared with those elicited by the known
potent phosphodiesterase inhibitor 1-methyl-3-isobutyl-xanthine (MIX). Included in this study was an examination of compound I's effects on the response of isolated bronchial rings from horseradish peroxidase (HRP)-sensitized rabbits subsequent to antigen challenge as well as its potential to attenuate or block the response to histamine in the isolated bronchial rings of control animals.

Also, an in vivo study was conducted on anesthetized California rabbits (control and HRP-sensitized animals) to determine the efficacy of compound I in altering respiratory changes associated with histamine or antigen-induced systemic anaphylaxis (i.e., lung resistance and dynamic compliance).

Finally, a study was made of crude cyclic AMP-phosphodiesterase enzyme preparations from human lung tissue and rabbit bronchial smooth muscle to determine if, in fact, compound I actually inhibited the cyclic AMP-phosphodiesterases of pulmonary tissue and bronchial smooth muscle. If demonstrated, this inhibition could explain, at least in part, the effects of compound I on bronchial smooth muscle.
CHAPTER 2

MATERIALS AND METHODS

Biological Specimens

California rabbits of either sex weighing between 2-3 kg were obtained from the Department of Animal Resources, University of Arizona. They were housed one per stainless steel cage and fed Wayne Rabbit Ration and tap water ad libitum. Sensitized California rabbits for the IgE anaphylaxis experiments were induced to synthesize anti-horseradish peroxidase antibodies only of the IgE class of immunoglobulin by means of the immunization schedule reported by Halonen et al. (1976). These animals were fed and housed under the same conditions as the control animals. Room temperature and humidity were set and maintained at 20-22°C and 55% respectively. All animals were allowed at least one week in the Department of Animal Resources animal care facility prior to use for experimentation.

Human lung tissue specimens for use in the cyclic nucleotide phosphodiesterase assay were obtained from patients suffering from lung carcinoma approximately one to two hours after surgery. Normal-appearing tissue was isolated and stored at -60°C in a Revco ultra-low freezer until use. These tissues were made available through the friendly cooperation of Doctors Michael White and Karen Steinbronn of
the Veteran's Administration Hospital Surgical Pathology Department, Tucson, Arizona.

**In Vitro Bronchial Ring Studies**

The methods of Pleisch and Calkins (1976) and Hooker et al. (1977) were employed in these investigations which involved the drug-induced responses of rabbit bronchi and intrapulmonary bronchioles.

Male or female California rabbits at a weight of 2-2.5 kg were killed by cervical dislocation and bled by severing the jugular vein. The lungs with trachea, esophagus, and heart attached were quickly excised and placed in aerated Kreb's-bicarbonate buffer (pH: 7.4) of the following composition (millimoles/liter): KCl 4.6, CaCl$_2$.2H$_2$O 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$.7H$_2$O 1.2, NaCl 118.2, NaHCO$_3$ 24.8, and Dextrose 10.0. The esophagus, heart, and trachea at its juncture with the main bronchi were separated from the lungs and discarded. 2-3 mm-wide rings of the main bronchi (right and left) were isolated. Smaller diameter airways (intrapulmonary bronchioles) were prepared as rings by removal of a 3 mm-wide section of the right and left lung at the mid-lung level, thus obtaining a cross-section of each lung 3 mm in width. Bronchioles could be differentiated from pulmonary vessels by submerging the
lung in buffer after removal of the lower lobe and gently applying pressure to the lung surface. In this way the vessels were established as distinct from the bronchioles since blood was exuded from the pulmonary vessels whereas air bubbles were readily observed exiting the bronchioles in response to applied pressure. Upon identification of the bronchiolar tissue a thin stainless-steel wire was placed through the center of the bronchiole and embedded in a piece of paraffin which was secured to the bottom of the dissecting dish. This held the tissue in place and simplified the removal of surrounding parenchyma (figure 4a). Using this technique, at least four intrapulmonary bronchiolar rings and up to four main bronchial rings could be prepared from each animal. Each individual ring was then transferred to tissue supports constructed of thin stainless-steel wire and suspended in a 10 ml isolated tissue bath containing Kreb's-bicarbonate buffer under constant aeration with a mixture of 95% O₂ and 5% CO₂ (figure 4b). Temperature was maintained at 37°C with a Radiometer type VTS 13c constant-temperature circulating water unit and contractions were measured isometrically (defined as a contraction which occurs when the muscle supports a load in a fixed position or attempts to move a load that is greater than the tension developed by the muscle: Vander et al., 1980) with a Statham UC3 force-displacement transducer and recorder on an Electronics
Figure 4a. Isolation of intrapulmonary bronchioles for \textit{in vitro} tissue bath studies.

Figure 4b. Suspended intrapulmonary bronchiolar ring.
for Medicine DR8 oscillographic recorder (White Plains, N.Y.) as changes in grams of force. Optimal tension for the main bronchial rings and intrapulmonary bronchioles was 2 g and 0.75 g respectively. The tissues were allowed to equilibrate for at least one hour at their respective optimal tensions and the bath fluid was replaced at 20 minute intervals with 37°C buffer during this time. Cumulative contractile and relaxant dose-response curves were determined for various agonists. Relaxation was measured in tissues contracted with carbachol at approximately twice the ED$_{50}$. Relaxant responses to compound I and 1-methyl-3-isobutylxanthine (MIX) were expressed as percent relaxation of the contracted tissue back to precontracted baseline (i.e. initial optimal tension level). Maximal constriction was achieved by bringing the final carbachol concentration in the tissue bath to 1 mM. Attenuation of the response to histamine (constriction) by compound I was determined in the intrapulmonary bronchioles by incubating these tissues with increasing concentrations of compound I for 5 minutes and then challenging the tissues with histamine (100 μM). Responses to histamine given alone were compared with responses to histamine after incubation with compound I.

Intrapulmonary bronchioles were obtained from horseradish peroxidase-sensitized animals and prepared for assay in tissue baths as described previously. The purpose of
these studies was to determine whether or not compound I would effectively alter the response of sensitized tissues to antigen (horseradish peroxidase) in the in vitro system under study. Tissues were isolated, suspended, and stretched optimally to 0.75 g and allowed to equilibrate. Tissue pairs which were anatomically similar were compared by incubating one tissue with compound I (140 μM) for 5 minutes while its pair received the same volume of 0.9% saline. After 5 minutes, tissues were challenged with horseradish peroxidase (500 μg/ml) and responses recorded over time. Maximal constriction was determined by the addition of carbachol (1 mM) following completion of the response to antigen.

All chemicals used were reagent grade. Drugs were made up in 0.9% saline and were added to the baths in volumes <1 ml. Additions were calculated on the basis of final concentration of drug in the 10 ml bath. Drugs included; carbamylcholine chloride (carbachol), histamine dihydrochloride, and 1-methyl-3-isobutylxanthine (Sigma Chemical Co., St. Louis, MO.). 2, 4-Diamino-5-cyano-6-bromopyridine (comp I) was synthesized according to Carboni et al. (1958) and kindly provided by Dr. Christoph De Häen, Division of Endocrinology, University of Washington, Seattle. Horseradish peroxidase type II was purchased from
Sigma. It was prepared in pyrogen-free 0.85% sodium chloride, sterilized by millipore filtration and stored at -20° C.

Cyclic Nucleotide Phosphodiesterase Assay

The method which was employed exclusively in assaying cyclic AMP phosphodiesterase activity and inhibition in rabbit and human lung and bronchial tissue was that of Thompson and Appleman (1971) with some minor modifications. Briefly, tritiated substrate was converted by tissue phosphodiesterase and the 5'-nucleotidase of cobra venom to \(^{3}\text{H}\)-adenosine. Product nucleosides were then separated from unreacted substrate by ion-exchange chromatography and the eluate counted for tritium on a liquid scintillation counter (figure 31). The limits of this method are determined by the specific activities of substrate isotopes and are 10 nM for cyclic AMP phosphodiesterase and 30 nM for cyclic GMP phosphodiesterase using the published assay procedure and a 0.4 ml reaction volume.

Enzyme samples were prepared from fresh human lung and rabbit bronchi by homogenization of a known weight of tissue diluted 1:5 (W:V) with homogenization buffer of the following composition: Tris hydrochloride (pH:8) 10 mM, bovine serum albumin (pH:8) 0.5 mg/ml, EDTA 4 mM, EGTA 1 mM,
tosyl-lysine-chloromethyl-ketone (TLCK) 20 mM, 2-mercaptoethanol (EME) 3.75 mM, and sucrose 0.32 M. Homogenization was carried out in a Wheaton 15 or 30 ml glass homogenizer using a Black and Decker 3/8" variable speed drill with care taken to maintain the tissues at low temperatures by keeping the glass homogenization unit in contact with an ice bath during homogenization. Drill speed was accurately controlled with a Staco type 3PN 1010 variable speed autotransformer.

The homogenate was then centrifuged at 10,000 rpm in a Beckman Model J2-21 centrifuge for 25 minutes at 4°C. Prior to centrifugation a 100 µl sample of the original homogenate was aliquotted and saved for assay of phosphodiesterase activity. The supernatant fraction was decanted and placed on ice and the pellet fraction was resuspended in homogenization buffer to the original, pre-centrifugation volume. During the initial studies, all three fractions (homogenate, supernatant, and pellet) were assayed for cyclic AMP phosphodiesterase activity. However it has been reported that 80-90% of the phosphodiesterase activity in human lung is present in the soluble fraction (Bergstrand, 1978), and this observation was found to be consistent with our studies. For this reason kinetic and inhibition studies were limited to the supernatant fraction. Dilutions were then made of the various fractions at 30,
20, 10, 5, 2, and 1 microliter of enzyme per ml of homogenization buffer in order to determine linearity of enzyme velocity with respect to enzyme concentration and time which was found to prevail if hydrolysis of added substrate was < 25% and reaction times were kept short (15 min or less). The assay was carried out as follows: 100 μl of the appropriate enzyme dilution was added to a reaction vessel containing 100 μl of assay buffer and 200 μl of homogenization buffer and incubated for the appropriate time (2, 5, or 10 minutes) at 30°C in a Dubnoff Precision metabolic incubator (Chicago, Illinois). Assay buffer consisted of the following: Tris-Cl 40 mM, MgCl₂ 20 mM, 2-mercaptoethanol (BME) 15 mM, 8-[^3H]-cyclic adenosine monophosphate =100,000 cpm/assay (= 5 pMoles), cyclic AMP 0.25 - 100 μM, bovine serum albumin 1.8 mg/ml, and distilled deionized water which was added to adjust for these final concentrations. Following incubation, reaction vessels (12 X 75 mm glass culture tubes) were removed and placed in a boiling water bath for 45 seconds to terminate the reaction. Snake venom 5'-nucleotidase (Ophiophagus hannah) was then added (0.05 mg/assay) to each reaction vessel in order to convert the hydrolyzed cyclic nucleotides to the appropriate nucleosides (i.e. ^3[H]-adenosine) and this mixture was again incubated for 10 minutes at 30°C. Following this incubation step, 1 ml of reagent grade methanol was added to each
reaction vessel and the entire contents of each vessel was pipetted into individual anion-exchange columns (Bio Rad AG 1-X2, 200-400 mesh resin diluted 1:4 in methanol) made by adding 1 ml of the methanol-resin slurry to a pasteur pipet (5 3/4" L. X 7.0 mm O.D., heavy wall glass). The mixture was allowed to elute into 20 ml glass liquid scintillation vials and another 1 ml of methanol was placed over each column to completely elute converted nucleosides. 8 ml of Aquasol (New England Nuclear, Boston, MA.) was added to each vial and the radioactivity of the combined eluate was determined by liquid scintillation spectrometry on a Beckman LS-3133P liquid scintillation counter. Samples were counted for 2 minutes (pre-set error of 2%). The velocity of hydrolysis was calculated by the following equation:

\[
\text{Velocity (pM/assay/min)} = \frac{\text{CPM (Measured-bkgd.)} \times \text{total substrate conc. (pMoles)}}{\text{CPM (Max-bkgd.)} \times \text{reaction time (min)}}
\]

The protein content of the enzyme samples was determined according to the method of Lowry et al. (1951).

Kinetic and inhibition studies were carried out on the supernatant fraction from human lung tissue homogenates and from homogenates of rabbit bronchiolar smooth muscle. These studies were conducted in an analogous fashion to the previously described phosphodiesterase assay with several
exceptions. For kinetic analyses a constant enzyme concentration was used with varying substrate concentrations in order that the apparent Michaelis-Menten constant (Km) and the maximal velocity of the enzyme (Vmax) could be determined from Lineweaver-Burk plots (Dixon, 1953 and Lineweaver-Burk 1934). Standard cyclic AMP concentrations were prepared from a stock solution that had been calculated to an exact concentration spectrophotometrically on a Beckman Model 25 spectrophotometer (the extinction coefficient of cyclic AMP at pH 7 is 14.650 at λmax = 259 nm). From this stock standard the following cyclic AMP concentrations were prepared for kinetic studies (micromoles/liter): 100, 50, 32.5, 25, 18, 16.25, 14.375, 12.375, 10, 7.875, 6.5, 5.0, 3.94, 3.25, 2.5, 2.0, 1.625, 1.25, 1.0, 0.875, and 0.8125. This range of substrate concentrations has been suggested to afford the most useful and meaningful spectrum for differentiation of low Km enzyme activity versus the mixture of cyclic nucleotide phosphodiesterase activities present in most tissues (Thompson et al., 1974).

Inhibitor studies with compound I and other known cyclic AMP phosphodiesterase inhibitors (1-methyl-3-isobutyl-xanthine) were conducted as described previously at a chosen substrate and enzyme concentration. Therefore, a typical assay mixture would contain 100 μl of the inhibitor at a chosen concentration, 100 μl of the enzyme preparation at a
chosen dilution, and either 200 μl of homogenization buffer or 100 μl of various substrate concentrations ranging from 0.25-100 M and 100 μl of distilled water. For the inhibitor studies as for the kinetic determinations reaction times were 10 minutes.

All chemicals used were reagent grade. 8-3[H]-cyclic 3', 5'-adenosine monophosphate (specific activity = 21 Ci/mMole) was obtained from Schwartz/Mann (Spring Valley, New York) as was cyclic AMP. Labelled nucleotides were purified by anion exchange chromatography using Bio Rad AG 1-X2, 200-400 mesh resin in columns prepared from pasteur pipets. Eluted samples with the highest counts were pooled and further diluted in reagent grade ethanol and stored at -20°C. Snake venom 5'-nucleotidase (Ophiophagus hannah, king cobra) was purchased from Sigma Chemical company (St. Louis, MO.) and made up as 0.5 mg/ml solutions in distilled water. This preparation could be stored at 4°C for several weeks. Anion-exchange resin (Bio Rad AG 1-X2, 200-400 mesh, chloride form) was washed extensively in 0.5 N NaOH, 0.5 N HCL and deionized water to a final pH of 5.0 and stored in the cold room.

In Vivo Anesthetized Rabbit Studies

These studies were undertaken to determine the efficacy of compound I in preventing or attenuating systemic anaphylaxis associated with parenteral challenge with either
histamine (control animals) or horseradish peroxidase (sensitized animals) in the anesthetized rabbit. The method which was used to assess the particular physiological parameters associated with anaphylactic alterations was that of Halonen et al. (1980). Anaphylaxis is characterized by a spectrum of physiological changes predominantly associated with respiration, circulation, and intravascular changes. Respiratory changes include a decrease in dynamic lung compliance ($C_{L\text{dyn}}$), an increase in total pulmonary resistance ($R_L$) and a transient apnea preceded in some rabbits by a short period of rapid, shallow breathing. The circulatory changes include an increase in mean pulmonary arterial pressure and systemic hypotension (Halonen, 1976). Intravascular alterations include the rapid development of basopenia and abrupt, transient thrombocytopenia and neutropenia (Halonen et al., 1973 and Pinckard et al., 1977).

Using the following method such changes could be recorded and quantified.

2-3 Kg rabbits were anesthetized by intravenous injection of 30 mg of sodium pentobarbital (Nembutal) per kg via a 23 3/4, 12" tubing butterfly infusion set (Abbot Hospitals Inc., No. Chicago, IL.) in the marginal vein of the ear. Nembutal was added in small doses as necessary throughout the experiment to maintain light surgical anesthesia. Following tracheostomy, a tracheal cannula was
inserted and connected to a heated Hewlett-Packard Fleisch type 00 pneumotachygraph and a Hewlett-Packard 7304 A respiratory flow transducer to measure breathing frequency and flow. Transpulmonary pressure was obtained by insertion of a polyethylene catheter with side holes and a closed end into the right pleural space with a 3 ml pneumothorax. This catheter was connected to a Hewlett-Packard P270 differential pressure transducer (the other arm of which was connected to a small bore tap from the tracheal cannula). Dynamic lung compliance ($C_{L_{dy}}$) and lung resistance ($R_L$) were obtained using a Hewlett-Packard 8816 A respiratory analyzer. To obtain phasic aortic pressure and right ventricular pressure, saline-filled polyethylene catheters were placed in the right ventricle and thoracic aorta threaded via the femoral vein and artery respectively and Statham P23Db pressure transducers were attached to these catheters. Heart rate was measured by an electrocardiograph (Lead I) obtained via subcutaneous needle electrodes. At the completion of these surgical procedures 1,000 U of sodium heparin per Kg were injected intrarterially. Circulatory data were recorded continuously on an Electronics for Medicine VR6 recorder and respiratory data were recorded on a Hewlett-Packard 7754 A recorder (figure 5).

After surgical placement of monitoring devices a 5-10 minute control period was recorded. This was termed
Figure 5. In vivo set-up for monitoring respiratory and cardiovascular responses in the anesthetized rabbit. $P_{Ao}$ = aortic pressure catheter; $P_{PA}$ = right atrial pressure catheter; $P_{TP}$ = trans-pleural pressure; $P_{PL}$ = intra-pleural pressure; $P_m$ = pressure in the mouth; $f$ = breathing rate; $V$ = flow rate; and $V_T$ = tidal volume. Dynamic lung compliance ($C_L$) and lung resistance ($R_L$) were mathematically derived from $P_{TP}$, $f$, $V$, and $V_T$. Adapted from Halonen et al. (1980).
the "pre-challenge" period and measurements were taken at one-minute intervals. This "pre-challenge" period was monitored in all experiments. At this point the protocol used for control animals challenged with histamine differed slightly from that which was used for sensitized animals challenged with horseradish peroxidase. In the procedure which was used for control animals, a saline infusion was administered via the marginal ear vein prior to the first histamine challenge. This infusion served as a control (i.e., compound I was infused in a 0.9% saline vehicle under the same infusion parameters subsequent to the first histamine challenge). In this manner, each animal served as its own control. A Harvard Apparatus infusion/withdrawal pump (Millis, MA.) was used and set at 30-80% (depending on syringe diameter) and a motor speed of 5 to deliver either the control saline vehicle or compound I in saline at a constant infusion rate of 1 ml/min. Length of infusions ranged from 10 to 50 minutes depending on the desired volume of infusate to be delivered. Within 5-10 minutes after "pre-challenge" infusion a 1 ml bolus of histamine (250 μg/ml) dissolved in physiological saline at pH 7.0 was delivered rapidly via the marginal ear vein and physiological alterations were recorded at 15 second intervals for the first 2.5 minutes, 30 second intervals to 5 minutes, and then every
minute for the duration of the response or until recovery from the challenge was apparent. Based on this procedure responses to histamine pre and post-compound I could be quantified and compared.

The sensitized rabbit studies were conducted in an analogous fashion to studies on control animals with several exceptions. A control period of 10 minutes was recorded, however the saline control-vehicle infusion step was bypassed and instead compound I infusion followed. Horseradish peroxidase (HRP), the antigen to which sensitized animals were producing IgE antibodies, was administered within 20-40 minutes following compound I infusion. HRP (10 mg/ml) was given as a 0.5 ml bolus via the intra-arterial catheter and responses were monitored chronologically in the same manner as responses of control animals to histamine. In these studies, sensitized animals could not serve as their own controls because of the fact that, having released their mediators, the cells responsible for mediating anaphylaxis are much less sensitive to subsequent antigen challenge and hence would not release a qualitatively equal concentration of mediators (Halonen et al., 1976).

A final study of both control and sensitized rabbits was undertaken to examine the effects of orally administered compound I in attenuating histamine and (or) HRP-induced
anaphylaxis. In this study compound I was administered via oral gavage as a suspension. 20-40 minutes following administration of compound I control animals were challenged with histamine (250 μg) and sensitized animals with HRP (0.5 mg) and responses were monitored as described earlier. This study was attempted in light of the findings by Johnson and De Haen (1979) that compound I was orally active in rats as an insulin secretagogue and that this action may involve cyclic nucleotide phosphodiesterase inhibition.

The following drugs were used in this study: heparin (1000 U/ml) was obtained from O'Neal, Jones, and Feldman (St. Louis, MO.); nembutal sodium solution from Abbot Laboratories (North Chicago, IL.); Xylocaine (lidocaine hydrochloride) 4% from Astra Pharmaceutical Products Inc. (Worcester, MA.); and pentobarbital sodium, euthanasia injection from Anthony Products Co. (Arcadia, CA.).
CHAPTER 3

RESULTS

In Vitro Bronchial Ring Studies

Initial dose-response curves were established for both primary bronchial and intrapulmonary bronchiolar rings which were isolated from rabbit lung. Responses to carbachol (figures 6 and 7) and histamine (figures 8 and 9) were recorded and $ED_{50}$ values established to provide the choice of optimal agonist concentration(s) against which compound I and methyl-isobutylxanthine (MIX) could later be tested for relaxant properties.

The primary bronchial rings responded in a similar manner as the smaller-diameter intrapulmonary bronchioles to either carbachol or histamine as indicated by the shapes of their dose-response curves and respective $ED_{50}$'s. However, the main bronchi appeared to be somewhat more sensitive to these agonists. In response to carbachol the $ED_{50}$'s were 0.67 μM and 0.85 μM for the primary bronchi and intrapulmonary bronchioles respectively. In response to histamine the primary bronchi had an $ED_{50}$ of 17.1 μM whereas the $ED_{50}$ for the intrapulmonary bronchioles was 35 μM.

The relaxant effect of compound I (figures 10 and 11) or methyl-isobutylxanthine (figures 12 and 13) on
Figures 6 and 7. Cumulative dose-response curves for isolated rabbit primary bronchi (fig. 6) and intrapulmonary bronchioles (fig. 7) to carbamylcholine chloride (carbachol). Response is percent of maximal constriction. Each point represents the mean ± S.E. for 6 animals.
Figures 8 and 9. Cumulative dose-response curves for rabbit primary bronchi (fig. 8) and intrapulmonary bronchioles (fig. 9) to histamine dihydrochloride. Response is percent of maximal constriction. Each point represents the mean ± S.E. of data obtained from 4 animals (primary bronchi), and 3 animals (intrapulmonary bronchioles).
Figures 10 and 11. Cumulative dose-response curves for rabbit primary bronchi (fig. 10) and intrapulmonary bronchioles (fig. 11) to 2,4-diamino-5-cyano-6-bromopyridine (compound I) following carbachol constriction (2 μM). Response is percent of maximal relaxation. Each point represents the mean ± S.E. of data obtained from 5 animals.
Figures 12 and 13. Cumulative dose-response curves for rabbit primary bronchi (fig. 12) and intrapulmonary bronchioles (fig. 13) to 1-methyl-3-isobutylxanthine (MIX) following carbachol constriction (2 μM). Response is percent of maximal relaxation. Each point represents the mean ± S.E. of data obtained from 5 animals.
previously constricted bronchial rings was investigated. Both compounds produced significant relaxation in either primary or secondary bronchial rings in a dose-related manner in tissues previously constricted with carbachol (2 µM). ED₅₀ values in response to compound I were 84 µM (primary bronchi) and 39 µM (intrapulmonary bronchioles). Methyl-isobutylxanthine relaxed these tissues in virtually the same manner and to the same degree as compound I with ED₅₀'s of 91 µM (primary bronchi) and 49 µM (intrapulmonary bronchioles). A comparison of these responses demonstrates that they are practically superimposeable (figures 14 and 15). Also noted was the fact that the intrapulmonary bronchioles were approximately 2 times more sensitive to the phosphodiesterase inhibitors (compound I and MIX) than were the primary bronchi as indicated by their respective ED₅₀'s.

Compound I's potential to attenuate or block bronchial smooth muscle constrictor responses to histamine was investigated in a study focusing on the intrapulmonary bronchioles which were incubated with varying concentrations of compound I for 5 minutes prior to challenge with histamine (100 µM). Compound I reduced the ability of these tissues to generate tension following histamine challenge as demonstrated in figures 16 and 17. At concentrations ≥ 10 µM compound I significantly reduced tension generation (p<.05) and at a 100 µM concentration, tension was approximately one-half that generated by histamine alone.
Figures 14 and 15. Comparison of the responses of rabbit primary bronchi (fig. 14) and intrapulmonary bronchioles (fig. 15) to compound I and MIX. Responses are percent of maximal relaxation.
Figure 16. Grams of tension generated by rabbit intrapulmonary bronchiolar rings in response to histamine challenge (100 μM) after incubation with compound I for 5 minutes at various concentrations. Each point represents the mean ± S.E. of 6 or more experiments. Values which are significantly different from control are indicated, a(p < .05), and b(p < .001).
Figure 17. Response of rabbit primary bronchi and intrapulmonary bronchioles to histamine dihydrochloride (100 µM). Intrapulmonary bronchiolar responses are recorded in the presence or absence of compound I at 1 µM and 100 µM final concentrations. The plot of primary bronchial response is for control group only. The response is grams of tension generated. Each point in the control groups represents the mean ± S.E. for 5 animals. In the compound I-treated samples, each point represents the mean ± S.E. of 4 experiments. Values which are significantly different than controls are indicated, *(P < .05), and ▲(*P < .001).
Figure 18. Response of rabbit intrapulmonary bronchioles to antigen (HRP). Tissues were from animals which had been sensitized to horseradish peroxidase (HRP). The response is percent of maximal constriction as determined by the addition of carbamylcholine chloride (1 mM). Compound I-treated tissues were incubated with 140 μM/l of compound I for 5 min. prior to antigen challenge (500 μg ml⁻¹ HRP). Each point represents the mean ± S.E. for data obtained from 6 animals. Values significantly different than controls are indicated, *(p < .05).
Finally, compound I significantly reduced the constrictor response of sensitized intrapulmonary bronchioles to antigen (figure 18). This study utilized tissues from animals which had been sensitized to horseradish peroxidase (HRP). Tissues were incubated with compound I (140μM) for 5 minutes and then challenged with antigen (HRP). It was apparent from this study that compound I reduced tension generation in sensitized tissues responding to antigen. However, the mechanism by which this response was attenuated remains unclear and may involve the cyclic AMP-dependent inhibition of mediator release in addition to smooth muscle relaxation.

Cyclic AMP-Phosphodiesterase Inhibition Studies

Prior to enzyme kinetic studies it was necessary to determine the presence of phosphodiesterase(s) in the tissues being studied as well as to establish that the velocity of substrate hydrolysis was linear with respect to time and enzyme concentration. These studies utilized the supernatant fractions of either bronchial smooth muscle homogenates from the rabbit or human peripheral lung tissue. Figures 19 and 20 demonstrate that velocity was indeed linear given the range of enzyme concentrations employed with respect to time at a constant substrate concentration (0.25μM cyclic AMP). Coincidentally, it was observed that, at a given protein concentration, there was more enzyme activity in rabbit
Figure 19. Velocity of hydrolysis of 8-[^3H]-3',5'-cyclic adenosine monophosphate by the cyclic AMP-phosphodiesterase(s) present in the supernatant fraction (10 K) of California rabbit bronchial smooth muscle homogenates. Linearity of velocity with respect to time and enzyme concentration is demonstrated. Substrate concentration was 0.25 μM cyclic AMP. Each point represents the mean of triplicate samples. The correlation coefficient for each line is, \( r \geq 0.95 \).
Figure 20. Velocity of hydrolysis of $8^{-3}[H]-3'$,5'$'$-cyclic adenosine monophosphate by the cyclic AMP-phosphodiesterase(s) present in the supernatant fraction (10 K) of human lung tissue homogenates. Linearity of velocity with respect to time and enzyme concentration is demonstrated. Substrate concentration was 0.25 μM cyclic AMP. Each point represents the mean of triplicate samples. The correlation coefficient for each line is, $r \geq 0.99$. 
bronchial smooth muscle (approximately two times as much) than in human peripheral lung tissue. This may have been due to the possibility that smooth muscle has inherently higher levels than peripheral lung tissue of either cyclic AMP-phosphodiesterases in general, or more of the high affinity (low $K_m$) form of the enzyme in particular, or both. It may also involve the fact that the human tissues were from elderly patients suffering from lung carcinoma and whose enzyme systems may, in some way, have been compromised. Finally, the explanation could conceivably be as simple as species variation in the distribution of cyclic nucleotide phosphodiesterases.

Kinetic analysis of rabbit bronchial smooth muscle cyclic AMP-phosphodiesterase (figure 21) demonstrated that compound I was an apparent competitive inhibitor of the high affinity enzyme in the soluble fraction of this tissue. Based on the linearity ($r = 0.99$) of a replot of the slopes obtained from the Lineweaver-Burk plot versus inhibitor concentration, the competitive inhibition produced by compound I was determined to be linear competitive inhibition. The $K_m$ of this enzyme was approximately 1.6 μM, and Vmax was 260 pM/mg protein/minute. The apparent $K_i$ for compound I was calculated from a Dixon plot to be approximately 75 μM (figure 32).
Figure 21. Lineweaver-Burk double reciprocal plot demonstrating apparent competitive inhibition of the low $K_m$ cyclic AMP phosphodiesterase enzyme present in the supernatant fraction (10 K) of rabbit bronchiolar smooth muscle homogenates with increasing concentrations of compound I. Protein content was 0.037 mg/assay.
Figure 22. Inhibition of human lung tissue cyclic AMP phosphodiesterase by either compound I or methyl isobutylxanthine. Protein content was .03 mg/assay. Substrate concentration (cyclic AMP) was 0.25 μM, and reaction time was 10 min. Each point represents the mean ± S.E. of 5 experiments (comp. I), and 3 experiments (MIX). Values which are significantly different than controls are indicated, *(p < .05), and ▲ (p < .001).
Finally, IC₅₀ values were determined in human lung tissue containing cyclic AMP-phosphodiesterase(s). The IC₅₀ value, which is the concentration of enzyme inhibitor required to produce 50% inhibition of the enzyme, was determined for compound I and methyl-isobutylxanthine. Both compounds significantly inhibited the enzyme(s) at all concentrations used (10-100μM). Methyl-isobutylxanthine proved to be at least 5 times more potent an inhibitor of the enzyme than compound I and had an IC₅₀ <10 μM while compound I had an IC₅₀ of approximately 50 μM (figure 22).

**In Vivo Studies in the Anesthetized Rabbit**

To determine the efficacy of compound I in attenuating or blocking systemic anaphylaxis in the rabbit in response to either histamine or antigen, several in vivo studies were carried out. Based on a limited knowledge of the pharmacokinetics of this compound and its low solubility, it became necessary to determine the optimal route of administration for the anesthetized rabbit. In the initial studies the compound was delivered as an i.v. infusion in which it was dissolved in physiological saline. A number of physiological parameters were measured. However, the two parameters of greatest significance to us were lung resistance (R_L) and dynamic lung compliance (C_Ldyn). The data obtained from four animals has been listed in table 1 as a comparison of responses (R_L and C_Ldyn) before and after compound I treatment at various concentrations and by various
TABLE 1.

Results of in vivo studies; lung resistance ($R_L$) and dynamic compliance ($C_{Ldyn}$) measurements.

<table>
<thead>
<tr>
<th>Percent of Pre-Challenge Value</th>
<th>1&lt;sub&gt;a&lt;/sub&gt;</th>
<th>2&lt;sub&gt;b&lt;/sub&gt;</th>
<th>3&lt;sub&gt;c&lt;/sub&gt;</th>
<th>4&lt;sub&gt;d&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>427</td>
<td>526</td>
<td>641</td>
<td>287</td>
</tr>
<tr>
<td>Saline</td>
<td>347</td>
<td>217</td>
<td>165</td>
<td>470</td>
</tr>
<tr>
<td>Comp. I</td>
<td>641</td>
<td>287</td>
<td>347</td>
<td>217</td>
</tr>
<tr>
<td>Control</td>
<td>470</td>
<td>328</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$R_L$: (maximum)

$C_{Ldyn}$: (minimum)

<sup>a</sup>Compound I administered via i.v. infusion (322 µg/Kg); control rabbit number 1; histamine challenge (75 µg/Kg); Saline was infused as a control vehicle since compound I was also made up in saline. Control period refers to histamine challenge prior to either saline or compound I infusion.

<sup>b</sup>Compound I administered by i.v. infusion (1.8 mg/Kg); control rabbit number 2; histamine challenge (74 µg/Kg)

<sup>c</sup>Compound I administered by oral gavage (54 mg); control rabbit number 3; histamine challenge (83 µg/Kg).

<sup>d</sup>Compound I administered by oral gavage (400 mg); HRP-sensitized rabbit number 4; HRP challenge (5 mg); control refers to a group of HRP-sensitized animals in response to antigen alone.
routes of administration (i.v. infusion or oral gavage). The numbers listed are the values for maximum resistance and minimum compliance obtained during each respective histamine or antigen challenge and they are listed as percent of pre-challenge values.

Based on results from the first experiment in which compound I was delivered i.v. through the marginal ear vein it was apparent that, at the chosen concentration (322 µg/Kg), compound I did not significantly affect the histamine-induced responses (figures 23 and 24). That is, the maximal lung resistance was 18% above control whereas the minimum compliance value was 31% below control.

In the second rabbit (figures 25 and 26), compound I was also delivered i.v. but at approximately 5.5-times the concentration of that given to the first animal. Although maximum resistance was 17% greater than the control response, the minimal compliance measurement was higher than control (12%) indicating that the compound may have produced an effect at this concentration.

In the next experiment, compound I was administered orally by gavage (54 mg bolus). Clearly, maximum resistance was reduced following this treatment in response to histamine challenge. The resistance measurement (maximum) was 24% lower than that of control. Compliance was also affected positively. The minimal compliance value, in response
Figure 23. Lung resistance measurements of the anesthetized rabbit following histamine challenge (75 μg/kg). Control response is compared with responses obtained following either a saline control infusion or compound I I.V. infusion (322 μg/kg). Measurements are plotted as percent of pre-challenge value.
Figure 24. Lung dynamic compliance ($C_{\text{dyn}}$) measurements recorded in the anesthetized rabbit following histamine challenge (75 µg/kg). The control response to histamine is compared with responses to either saline or compound I I.V. infusion (322 µg/kg). Measurements are plotted as percent of pre-challenge value.
Figure 25. Lung resistance measurements from the anesthetized rabbit following histamine challenge (74 μg/kg). Control response is compared to response obtained following compound I I.V. infusion (1.8 mg/kg). $R_L$ values are percent of pre-challenge values.
Figure 26. Lung dynamic compliance ($C_{L_{dyn}}$) measurements in the anesthetized rabbit following histamine challenge (74 $\mu$g/kg). The control response to histamine is compared to the histamine response following compound I infusion I.V. (1.8 mg/kg). Compliance measurements are as percent of pre-challenge value.
Figure 27. Lung resistance measurements in the anesthetized rabbit following histamine challenge (83 μg/kg). Control response is compared to response obtained following administration of compound I by oral gavage (54 mg). $R_L$ values are percent of pre-challenge value.
Figure 28. Lung dynamic compliance ($C_{L_{dyn}}$) measurements recorded in the anesthetized rabbit following histamine challenge (83 $\mu$g/kg). Control response to histamine is compared with response to histamine following compound I administration by oral gavage (54 mg). $C_{L_{dyn}}$ values are percent of pre-challenge value.
to histamine challenge following compound I administration, was 22% above that of control (figures 27 and 28).

Finally, the effect of compound I in preventing or attenuating anaphylaxis in the sensitized rabbit following antigen challenge was investigated (figures 29 and 30). Compound I was administered by oral gavage (400 mg), and the animal was then challenged with antigen (horseradish peroxidase, 5 mg). The results indicate that compound I attenuated the increase in maximum resistance by up to 30%. However, the minimal compliance measurement was slightly below control (=9%).
Figure 29. Lung resistance measurements ($R_L$) in the sensitized rabbit. The animal was sensitized to horseradish peroxidase (HRP). Response is following antigen (HRP) challenge (5 mg). Control response to HRP is compared with response to antigen following compound I via oral gavage (400 mg). $R_L$ values are percent of pre-challenge value.
Figure 30. Dynamic compliance measurements in the sensitized rabbit in response to antigen challenge (HRP, 5 mg). Control response to antigen is compared with antigen response following compound I administration by oral gavage (400 mg). $C_L$ values are percent of pre-challenge value.
Figure 31. Radio-isotopic method used for determining the inhibition of cyclic nucleotide phosphodiesterases. Adapted from Thompson, W., and Appleman, M. Cyclic nucleotide phosphodiesterase activities from rat brain. Biochem. 10;2, 1971.
Figure 32. Dixon plot determines the enzyme:inhibitor dissociation constant ($K_i$) of compound I using two inhibitor concentrations in rabbit bronchial smooth muscle low $K_m$, soluble cyclic AMP-phosphodiesterase. Substrate concentrations (cyclic AMP) are indicated. Apparent $K_i = 75 \mu M$. 
The sequence of experiments which were conducted in the investigations of compound I's pharmacodynamic role in bronchospasm, and which were based on the general observations that this compound inhibited cyclic nucleotide phosphodiesterases in dog kidney and rat pancreatic islet tissues were as follows;

1. Determination of the molecular basis of action for any drug or compound is certainly of potential significance; however, such a determination should logically follow an observed physiological effect of the agent in question. Based on this rationale, initial studies with compound I focused on its ability to relax bronchiolar smooth muscle in vitro in a system which utilized isolated rabbit bronchi.

2. Subsequent to the finding that compound I did, in fact, exert a fairly potent bronchodilatory effect in the in vitro system studied, in vivo studies were initiated.
3. Based on results from both in vitro and preliminary in vivo studies with compound I, and the further realization that this compound might indeed prove to have a potential therapeutic value, studies into its molecular basis of action were undertaken. These studies focused exclusively on the inhibition of pulmonary phosphodies­
terases from rabbit bronchial smooth muscle and human peripheral lung tissue by compound I.

4. Finally, in order to relate the observed physiological response (bronchodilation) to the hypothesized molecular mechanism of action of com­
 pound I (cyclic AMP-phosphodiesterase inhibition), a comparison was made with a drug (methyl­
isobutylxanthine) having physiologically similar bronchodilatory actions and whose molecular basis of action was hypothetically analogous, at least in part, to that of compound I.

In Vitro Studies

The in vitro system employed in these studies utilized rings of rabbit main bronchi and intrapulmonary bronchioles and was similar to that described by Hooker et al., (1977). The use of isolated airway segments from a variety of experimental animals as well as from humans in the study of drug action on airway caliber has been a
commonly employed practice for many years. Earlier studies made use of tracheal rings which were tied together to form a chain (Castillo and DeBeer, 1947), spirally-cut tracheal or bronchial strips (Patterson, 1958), or bronchial rings attached end to end after sectioning and removal of their cartilagenous parts (Akcasu, 1959). The method described by Hooker et al. is a modification of that which was originally introduced by Bevan (1962). These investigators explained that the use of individual bronchiolar rings may provide greater accuracy than earlier techniques in the interpretation of responses with respect to drug interactions because generalizations, based on the large airway sections which lack pharmacologic uniformity, may be inaccurate (Brocklehurst, 1958, and Eyre, 1969). Also, it is generally believed that the trachea is not as important as it was once thought to be in terms of its influence on overall airway caliber in response to drugs or chemical mediators. Instead, the bronchioles are the tissues of most significance since drug-induced effects on the respiratory smooth muscle undoubtedly have their most pronounced action on these tissues (Fleisch et al., 1973).

Bronchoconstriction plays a major role in the early phase of the asthmatic response (Parker et al., 1965). It appears that the parasympathetic nervous system is extremely important in this regard, since it may affect the airways
via reflexes involving bronchial smooth muscle or increased mediator release. Vagal innervation is important in controlling normal bronchomotor tone as well as in modulating this tone in disease. When irritant receptors in airway epithelium are stimulated mechanically, pharmacologically, or chemically, a striking reflex bronchoconstriction is produced via the vagal pathways and this reflex may be exaggerated in asthma (Nadel, 1976). Adrenergic innervation appears to be less potent and blockade of beta-adrenergic receptors has little or no effect on healthy subjects, however it may cause bronchoconstriction in some asthmatic subjects as well as inducing a hypersensitivity to histamine, serotonin, acetylcholine, bradykinin, and slow-reacting substance of anaphylaxis (Szentivanyi, 1968).

Bronchial hyperreactivity occurs in asthma and may be due to prior bronchial obstruction, hyperplasia of smooth muscle, or abnormalities in the parasympathetic or sympathetic nervous systems. For example changes in lung function, which are characteristic of the early phase of asthma, caused by allergens or by non-specific stimuli, can be reproduced by aerosols of histamine or cholinergic agents such as carbachol and methacholine (Parker et al., 1965). In asthmatics, the amounts of these agents needed to produce lung changes is smaller than in normal subjects (Tiffeneau, 1955), thus demonstrating hyperreactivity of the airways.
These changes have been attributed to vagal activity because they are substantially reduced by atropine or by ganglionic blockade, and can be reproduced in animals by stimulating the vagus electrically (Poster, 1966).

Histamine has been shown to produce bronchoconstriction directly via $H_1$-receptors in bronchial smooth muscle (Ash and Schild, 1966), as well as via a vagally-mediated component. For example, the usual bronchoconstrictor response to systemically administered histamine or antigen was decreased by blocking vagal conduction (Karczewski and Widdicombe, 1969, Mills et al., 1969, and Mills and Widdicombe, 1970). On the other hand, the in vitro bronchoconstrictor effects of histamine can be neither abolished nor reduced by hexamethonium (Bouhuys et al., 1960) or atropine (DeKok et al., 1966) suggesting indirect involvement of cholinergic pathways. Also, vagal cholinergic stimuli promote airway muscle contraction. Atropine, which blocks the effects of cholinergic stimuli at the neuromuscular junction, inhibits many airway constrictor responses as well as decreasing smooth muscle tone in the airways. Therefore, decreased constrictor responses after atropine can be explained by assuming that the contractile agonist (i.e., histamine or other anaphylactic mediators which produce bronchoconstriction) elicits increases of vagal efferent stimuli via a reflex, possibly initiated by vagal sensory
receptors in the airway walls. These results have been interpreted to suggest that histamine initiates a reflex which reinforces its direct action on bronchial smooth muscle (Bouhuys, 1960, and Mills et al., 1969).

In view of the fact that histamine has been established as one of the major mediators of bronchoconstriction during anaphylaxis, and that this response in bronchial smooth muscle involves both direct and vagally-mediated components, an investigation of the effects of histamine and the potent congener of the parasympathetic neurotransmitter acetylcholine, carbachol, on isolated bronchiolar rings was undertaken.

The purpose of this study was two-fold. To establish a valid and consistent protocol in which to compare bronchial smooth muscle responses to bronchoconstrictors with compound I and other bronchodilators, it was necessary to determine ED\textsuperscript{50} values for these agonists. Following the characterization of these responses it was then necessary to correlate these with values (ED's\textsubscript{50}) found in the literature. Secondly, based on our objective which was to evaluate the potential bronchodilator activity of compound I, it was necessary to effectively "simulate" bronchoconstriction in the isolated bronchial preparations, either prior to or subsequent to exposure of these tissues.
to compound I, by the addition of "effective doses" of agonists. Since histamine and carbachol are known to each have different plasma-membrane receptors (histamine = H₁- and H₂-histaminergic, and carbachol = nicotinic - and muscarinic-cholinergic), and since both have been well established as bronchoconstrictor agents, we felt that a negative inotropic response in airway smooth muscle to compound I following carbachol or histamine constriction would strongly suggest the possibility that compound I was acting via some post-receptor event mechanism (i.e., phosphodiesterase inhibition) in contrast to a specific receptor antagonism.

The ED₅₀ values which were obtained in rabbit primary bronchi and intrapulmonary bronchioles responding to either carbachol or histamine were generally in agreement with values reported in the literature in which similar experimental procedures were used. The ED₅₀ values which we obtained in the rabbit in response to carbachol for the primary and secondary bronchi were 0.67 μM and 0.85 μM respectively (figures 6 and 7); ED₅₀'s of these same tissues in response to histamine were 17 μM (primary bronchi), and 35 μM (secondary bronchi) - figures 8 and 9.

Fleisch and Calkins (1976), using a similar method, obtained ED₅₀ values of 0.54 μM in the rabbit trachea and 0.70 μM in the bronchi of animals exposed to carbachol.
These values agree well with those which we obtained. In response to histamine, these investigators determined an \( ED_{50} \) of 8 \( \mu M \) in the rabbit bronchi, approximately one-fourth of the value which we obtained. One possible explanation for this difference may be the use of bronchial strips by Fleisch and Calkins in contrast to bronchial rings used here. Also, these researchers used a different strain of rabbits, which may suggest an inherent difference in histamine sensitivity.

With regard to the smaller diameter airways (intrapulmonary bronchioles) it was interesting that these tissues were approximately 10-30 times more sensitive to carbachol than to histamine on a molar basis in control (non-sensitized) animals. This difference in sensitivity has also been reported by others. These findings underline the importance of cholinergic influences via parasympathetic innervation relative to bronchomotor tone (described earlier), and more specifically to bronchoconstriction, especially when considering the fact that the airways of asthmatics have been characterized as hypersensitive to vagal input. Also, although histamine has been well established as an important mediator of bronchoconstriction during anaphylaxis, cholinergic activity to the respiratory tree during this response may be as important, if not more so.
Compound I or methyl-isobutylxanthine (MIX) significantly relaxed carbachol-constricted primary and secondary rabbit bronchi with respective ED$_{50}$ values of 84 and 91 $\mu$M (primary bronchi), and 39 and 49 $\mu$M (intrapulmonary bronchioles).

Compound I also significantly reduced the tension generated in intrapulmonary bronchiolar rings in response to a 100 $\mu$M histamine challenge (figure 16). The ability of MIX to produce a similar effect was not investigated.

Finally, compound I significantly attenuated the constrictor response of sensitized rabbit intrapulmonary bronchioles by reducing the tension generated in response to horseradish peroxidase (HRP), the antigen against which these animals were producing IgE antibodies.

MIX was chosen as the positive control because 1) MIX has been described as a potent cyclic nucleotide phosphodiesterase inhibitor with inhibitory activity on the order of 10-15 times greater than that of theophylline (Beavo et al., 1970). 2) MIX and theophylline are closely related methylxanthines differing structurally only in their number 3-positions in which theophylline contains a methyl group and MIX, an isobutyl moiety. Theophylline is one of the major drugs used in the treatment of asthma as indicated in a recent issue of The Medical Letter (September, 1982), and 3) theophylline and MIX have been firmly established as
potent bronchodilators whose effects have been at least partly related to the ability of this class of compounds (methylxanthines) to inhibit cyclic nucleotide phosphodiesterases (Kaliner and Austen, 1974, and Murad and Kimura, 1974). Since the proposed mechanism of action of compound I is cyclic AMP-phosphodiesterase inhibition it was felt that a similar physiological response between compound I and MIX would provide further evidence in support of this possibility.

The relaxant response of isolated bronchioles to compound I or MIX following carbachol constriction was dose-dependent and up to 80% relaxation occurred at a 100 μM concentration of either of these two compounds. Also, the dose-response curves for both the primary bronchi and intrapulmonary bronchioles to compound I and MIX were virtually superimposeable (figures 14 and 15). These results suggested the possibility that compound I and MIX might be acting by a similar "non-selective" (non receptor-dependent) mechanism to produce bronchial smooth muscle relaxation and, based on observations that both compounds significantly inhibited cyclic nucleotide phosphodiesterases, this provided further evidence which implicated phosphodiesterase inhibition as a potential mechanism responsible for producing the effects which were observed.
The logic which was used in considering these responses to be "non-selective" was based on the observations that agents, which selectively block and (or) selectively reduce the constrictor responses of isolated bronchial smooth muscle to carbachol such as the muscarinic-cholinergic receptor antagonist atropine, prevent constriction by a highly selective receptor-antagonistic mechanism and therefore are extremely potent at very low concentrations. Fleisch and Calkins (1976) demonstrated complete inhibition of bronchiolar constriction to a 0.5 \( \mu \text{M} \) carbachol challenge following incubation of bronchiolar strips with atropine (10\(^{-8}\) M). At a concentration of 2 \( \mu \text{M} \) carbachol (the concentration which we used to constrict isolated bronchiolar rings), atropine (10\(^{-8}\) M) pre-treatment resulted in a 70% inhibition of the constrictor response whereas, in our studies with compound I and MIX, a similar degree of inhibition required approximately 10,000 times this concentration of either compound on a mole-for-mole basis.

The response of intrapulmonary bronchiolar rings to histamine (100 \( \mu \text{M} \)) following pre-incubation with compound I (5 min.) was investigated. Compound I significantly reduced the amount of tension generated by these tissues but did not completely block the histamine-induced response over the dose-range tested (0.1-100 \( \mu \text{M} \)). These results appear to indicate that compound I again acted non-selectively (other
than through a receptor-mediated mechanism) to decrease muscle-tension generation in response to histamine. This response was characteristically different from responses of histamine-challenged tissues following incubation with classical antihistamines. For example, very low doses of antihistamines can completely block the response to histamine as well as significantly reduce responses to higher-doses of histamine. Fleisch and Calkins (1976) demonstrated that, with the addition of the $H_1$-receptor antagonist pyrilamine, isolated rabbit bronchiolar strips responded 59 times less than in the absence of the blocker. Their conclusion was based on the rightward-shift of the dose-response curve and the corresponding ED$_{50}$ values. In fact, these tissues were completely unresponsive to histamine at 50 $\mu$M in the presence of pyrilamine (0.1 $\mu$M) whereas only a 10% response (therefore 90% inhibition) was observed at a histamine concentration of 100 $\mu$M, the same histamine concentration which we used. In contrast, the greatest degree of inhibition of histamine-induced constrictor responses following incubation of tissues with compound I was approximately 50% at a concentration of 100 $\mu$M, 1,000-times the concentration of pyrilamine required to elicit a 5-fold greater inhibition.

The effect of antigen challenge on intrapulmonary bronchioles isolated from sensitized rabbits following
incubation with compound I was also investigated. The maximum tension generated subsequent to antigen challenge was 37% less in the presence of compound I (figure 18). It is important to note, however, that compound I did not completely block the response to antigen, indicating that the attenuation of tension generation caused by compound I was also apparently via some non-specific mechanism.

Mast cells, which lie in close proximity to airway smooth muscle cells, contain surface receptors with a high affinity for IgE antibodies (Ishizaka and Ishizaka, 1977). Subsequent exposure of the mast cell to the appropriate antigen or anti-IgE causes bridging across the IgE molecules and initiation of a series of intracellular biochemical changes leading ultimately to mediator release (figure 2). Evidence from peripheral leukocytes suggests that these events are controlled by cyclic AMP and probably cyclic GMP and can therefore be modified by drugs or hormones. Beta-adrenergic agonists, methylxanthines, and prostaglandin E increase cyclic AMP and inhibit mediator release (Tattersfield, 1979).

An explanation which appears to remain consistent with the finding that compound I reduced the antigen-induced constrictor response in vitro in sensitized rabbit airways is based upon one of two mechanisms, both of which are.
believed to be regulated by cyclic AMP as the common mediator. Inhibition of cyclic AMP-phosphodiesterase(s) would be expected to lead ultimately to smooth muscle relaxation in these tissues due to the accumulation of intracellular cyclic AMP. Alternately, compound I may have partially blocked the release of some of the mediators of broncho-constriction as it has been established that cyclic AMP regulates mediator release (Kaliner and Austen, 1974), although exactly how it does so remains unclear. Apparently mediator release was not completely blocked. Therefore it is felt that the most probable explanation for the inhibition of tension-generation by Compound I in these tissues is that of smooth muscle relaxation mediated via cyclic AMP-phosphodiesterase inhibition. This explanation is similar to that suggested for the smooth muscle responses which were observed in control tissues responding to either histamine or carbachol.

Several additional in vitro studies which would provide further evidence that the negative inotropic response of rabbit bronchial smooth muscle to compound I was mediated by the inhibition of cyclic AMP-phosphodiesterase have been considered. For instance, if this compound was inhibiting phosphodiesterase in the smooth muscle cells studied, the relaxant effects should be potentiated by beta-adrenergic agonists. Also, there exists the possibility
that compound I itself might have been interacting with the beta-adrenergic receptor as an agonist. Therefore, to determine this as a possible mechanism for compound I's action on bronchial smooth muscle, it would be necessary to incubate these tissues with a beta-adrenergic antagonist (e.g., propranolol) prior to the addition of compound I.

With regard to the attenuation of tension-generation following antigen challenge it is possible that compound I effectively reduced mediator release in addition to causing smooth muscle relaxation. This possibility could be investigated using a system of isolated sensitized mast cells, basophils, or platelets in which antigen-induced mediator release in both the presence and absence of compound I could be characterized.

Finally, it would be necessary to measure cyclic AMP levels directly in these tissues following exposure to compound I to determine whether or not cyclic AMP is elevated, and attempts would then have to be made to correlate cyclic nucleotide levels with the observed physiological changes.

**In Vivo Studies**

During the preliminary stages of evaluation and testing new compounds, *in vivo* experiments using appropriate animal models can provide invaluable insight into the likely therapeutic potential. *In vitro* studies are of value in the
elucidation of the mechanism of action of drugs in the particular target tissues studied, as well as for generalized drug-screening procedures. However, the extrapolation of \textit{in vitro} results to anticipated responses in the living animal is prone to error since it is impossible to anticipate all of the responses to a given agent in a particular organism based solely on experimental results obtained \textit{in vitro}. Therefore our aim was to characterize the effects of compound I \textit{in vivo} using an experimental model of asthma that would be consistent with our \textit{in vitro} model thereby allowing for cautious extrapolation of the \textit{in vitro} results.

The \textit{in vivo} model which was used was that of Halonen et al. (1980) in which respiratory alterations in the anesthetized rabbit in response to histamine or antigen could be recorded and quantified. The same species (California rabbit) was used in these studies as was employed in the \textit{in vitro} bronchial ring studies.

Dynamic lung compliance ($C_{L\text{dyn}}$) and lung resistance ($R_L$) measurements were determined for control animals following histamine challenge and sensitized animals following antigen challenge. The effectiveness of compound I in blocking or decreasing the response of these animals to a challenge capable of eliciting a characteristic anaphylactic response was investigated. These studies were preliminary
in nature and, as pointed out in the results section of this paper, it was necessary to establish an optimal route of administration which would allow us the flexibility to readily adjust concentrations and, more importantly, to determine the route by which compound I would have its most profound pharmacologic effects.

After two apparently unsuccessful attempts to reduce the response of control animals to histamine following different intravenous concentrations of compound I (figures 23-26), the decision was made to administer the compound orally by gavage. Figures 27 and 28 demonstrate that the response of this control animal to histamine was apparently reduced following compound I treatment. That is, the maximum lung resistance measurement following compound I gavage was substantially lower than the control value in response to histamine, and the minimal compliance value was greater than that of control. These results appear to indicate that compound I, when administered orally to the control anesthetized rabbit, effectively diminished the adverse respiratory changes associated with histamine challenge.

Based on these results it appeared that the oral route of administration was more effective than parenterally (i.v.) administered compound I, although this possibility should be interpreted cautiously since the "effective" oral dose was greater than either of the i.v. doses given to the first two control animals.
Since compound I was apparently successful in decreasing the histamine-induced response in a control animal following oral administration, we chose to examine the compound's ability to similarly attenuate the anaphylactic response of the sensitized rabbit to antigen. (Histamine is considered to be the major mediator responsible for lung resistance changes during antigen-induced anaphylaxis.)

The sensitized animal received 4-times the dose of compound I as the control animal prior to antigen challenge. The maximum lung resistance measurement following antigen challenge was 30% below that of control, whereas dynamic lung compliance measurements were essentially the same (figures 29 and 30). Again, it appeared that compound I was apparently effective in reducing some of the pathophysiological respiratory alterations characteristic of anaphylaxis. Although the reduction of these changes did not appear to be as great in the sensitized rabbit as they were in the control animal, it is important to realize that, whereas histamine was apparently the sole mediator of the observed responses in the control animal, in the antigen-challenged sensitized animal a variety of mediators are thought to be involved including histamine, slow reacting substance of anaphylaxis, eosinophil chemotactic factor of anaphylaxis, 5-hydroxytryptamine, as well as prostaglandins, bradykinins, and probably others.
Based on the small number of animals studied, and given the fact that wide variations of response are apt to occur among individuals within a population, these preliminary results can only be viewed as suggestive of the possibility that compound I was indeed effective. Further *in vivo* studies will be necessary to prove the efficacy of compound I in the treatment of anaphylaxis. Also, the results from the *in vitro* studies of rabbit bronchiolar ring preparations appear to be consistent with these *in vivo* results, again suggesting that compound I was acting non-selectively to prevent bronchiolar smooth muscle tension-generation in the anesthetized rabbit in response to histamine or antigen. This reduction of tension-generation in the airways of responding animals may have been responsible for the decreased lung resistance measurements obtained subsequent to compound I administration.

**Cyclic Nucleotide Phosphodiesterase Studies**

Having provided *in vitro* and *in vivo* results which appeared to suggest a pharmacological role for compound I in bronchial smooth muscle relaxation, a study of the effects of compound I on the inhibition of cyclic AMP-phosphodiesterase from rabbit bronchial smooth muscle and human peripheral lung tissue was undertaken.
Since compound I had previously been shown to inhibit the cyclic AMP-phosphodiesterase(s) from rat pancreatic islet tissue and dog kidney, and since cyclic AMP has been implicated as a major mediator of smooth muscle relaxation (Bar, 1974, Triner et al., 1971, and Adelstein, 1978), it seemed likely that the demonstration of phosphodiesterase inhibition in pulmonary tissues would provide further evidence that the observed physiological responses associated with compound I were mediated by the inhibition of the cyclic AMP-phosphodiesterase(s) and the resultant increase in intracellular levels of cyclic AMP in these tissues.

In general the system of enzymes collectively known as the cyclic nucleotide phosphodiesterases is an extremely complex family of enzymes with activity present in virtually all living cells. Some of the major characteristics of this system include:

a) the enzymes exist in several different molecular forms (Strada et al., 1974, Thompson and Appleman, 1971, and Monn and Christiansen, 1971);

b) these isozymes are unequally distributed among the various tissues (Weiss and Costa, 1968, Butcher and Sutherland, 1962, and Uzunov et al., 1974);

c) their kinetic properties and substrate affinities are variable (Rosen, 1970, Thompson and Appleman, 1971, Song and Cheung, 1971, Amer and Mayol, 1973, and Bergstrand, 1980);
d) they can be selectively activated by an endogenous protein (Weiss et al., 1974, and Levin and Weiss, 1976);

e) they can be selectively inhibited and activated by drugs (Uzunov et al., 1974, Fertel and Weiss, 1976, Weiss et al., 1974, and Wells et al., 1975);

f) they possess allosteric sites that can influence their activity (Russell et al., 1972, and Franks and MacManus, 1971).

Our investigations were limited to the study of the soluble, low $K_m$ (high affinity) pulmonary tissue phosphodiesterase for the following reasons:

1. Although there are phosphodiesterases associated with both particulate (membrane-bound) and soluble (cytosolic) cell fractions, 80-90% of the "total phosphodiesterase activity" present in human lung tissue, including a low $K_m$ form, was reported to be contained in the soluble fraction (Bergstrand, 1980),

2. The low $K_m$ (high affinity) isozyme is believed to be more critical than the high $K_m$ (low affinity) forms in terms of biological regulation and as a potential target for drug interactions (Amer and Kreighbaum, 1975). This is suggested based on
findings that the intracellular levels of the cyclic nucleotides are in the micromolar range or lower (Robison et al., 1968) and,

3. Weiss and Strada (1972) have suggested that it is the low $K_m$ form of the enzyme which is responsible for regulating basal cyclic AMP levels, whereas the high $K_m$ form(s) may be more important in regulating cyclic AMP levels following hormonal stimulation of the nucleotide cyclase. Since our studies focused on in vitro systems in which phosphodiesterase inhibition was evaluated relative to basal cyclic AMP levels (i.e., in the absence of hormonal stimulation), we studied the low $K_m$ form of the enzyme (Note: the substrate levels used in the assay largely determine which form of the enzyme is being assayed).

Enzyme kinetic studies of this low $K_m$ enzyme from rabbit bronchial smooth muscle intrapulmonary bronchioles were carried out for the purpose of determining whether or not compound I did in fact cause inhibition of this enzyme and, if so, to characterize the type of inhibition. In these experiments, preliminary indications suggested that compound I was an apparent competitive inhibitor of this enzyme (figure 21).
Following these studies, a comparison between the phosphodiesterase inhibiting capacity of compound I and MIX was made in the low $K_m$ soluble enzyme from human peripheral lung tissue. Both compound I and MIX significantly inhibited this enzyme; the potency of MIX being about 5-times greater than that of compound I (figure 22). The $IC_{50}$ value which was obtained for MIX ($=10 \mu M$) corresponded with that determined by Bergstrand et al. (1978). They used a similar assay procedure, and established an $IC_{50}$ value of $8 \mu M$ in a partially-purified low $K_m$ enzyme from the supernatant fraction of human lung tissue homogenates (at a substrate concentration of $0.13 \mu M$ cyclic AMP).

Based on these observations further evidence was provided which suggested that the observed in vitro physiological effects of compound I and MIX, and the in vivo effects of compound I, were related to the inhibition of cyclic AMP-phosphodiesterase. This "apparent" relationship is thought to involve smooth muscle relaxation in response to the inhibition of cyclic AMP-phosphodiesterase.

Evidence has been presented that suggests that phosphodiesterase inhibition may be the mechanism by which compound I produced the physiological effects which were observed in the rabbit. However, further studies will be necessary to confirm this. For example, the measurement of cyclic nucleotide levels in vitro as well as in vivo
in response to compound I, and the subsequent correlation of these levels with observed physiological changes would be a most convincing step towards proving this hypothesis.

Since the role of cyclic AMP in smooth muscle relaxation is presently based, in part, on speculation and extrapolation of isolated findings, an attempt to make excessively broad generalizations and (or) claims about specific molecular interactions between compound I, cyclic AMP, cyclic GMP, cyclic nucleotide phosphodiesterases, and the resultant physiological responses based on these interactions would be inappropriate. Certainly there are other possible explanations for the physiological effects which were observed in response to compound I.

Throughout this paper compound I has been compared with the methylxanthines on the basis of similarities in their proposed mechanism of action relative to their bronchodilatory effects. It has been the general belief for nearly 15 years that phosphodiesterase inhibition and the consequent cyclic nucleotide elevations represent the main mechanism for the effects of the methylxanthines. However, based on the initial findings by Afonso and O'Brien (1970) that aminophylline (theophylline ethylenediamine) inhibited the cardiovascular metabolic and hemodynamic effects of adenosine, and more recently that most of the actions of
adenosine are antagonized by theophylline and other methyl-
-xanthines (Fredholm, 1980) by apparent competitive inhibi-
tion of the adenosine receptor (Fain and Malbon, 1979, and 
Olson et al., 1976), as well as findings that two properties 
of theophylline; namely, inhibition of phosphodiesterase and 
antagonism of adenosine actions, might be dissociated 
(Smellie et al., 1979), it is evident that the decade-old 
view concerning the mechanism of action of the methylxan-
thines may no longer hold true. Of particular significance 
in view of our studies was the finding that adenosine poten-
tiates anaphylactic histamine release and that it causes 
contraction of respiratory smooth muscle. These effects 
were shown to be antagonized by theophylline in considerably 
lower concentrations than those required to inhibit cyclic 
AMP and cyclic GMP hydrolysis (Fredholm, 1980). 

In light of these findings there is, at present, 
no reason, other than structural dissimilarities with the 
methylxanthines, to discount a similar interaction of 
compound I with the adenosine receptor, and studies into 
this possibility may be warranted. This is not to imply 
that compound I has to necessarily act by a similar mecha-
anism as the methylxanthines. It is only to stress that 
there may be a variety of other actions of this compound 
which culminate in the physiological response observed,
namely the inhibition of tension-generation in bronchial smooth muscle following exposure to bronchoconstrictor agents.

**CONCLUSIONS**

A number of experimental findings which may indicate a potential therapeutic role for compound I as a bronchodilator have been discussed. Also, preliminary studies into the mechanism of action of this compound indicate that cyclic nucleotide phosphodiesterase inhibition may play a critical role in terms of the pharmacological action(s) of compound I.

The following is a listing which summarizes the conclusions derived from this work;

1. Compound I was demonstrated to have a potent bronchial smooth muscle relaxant effect on isolated rabbit airways following constriction of these airways with either carbachol or histamine.

2. Isolated rabbit bronchial smooth muscle, which had been previously constricted with carbachol, relaxed to methyl-isobutylxanthine in virtually the same manner and to the same degree as it did to similar molar concentrations of compound I, suggesting the possibility that these two compounds acted via a common biochemical or biophysical pathway to produce this response.
3. The response (constriction) of sensitized-isolated rabbit bronchial smooth muscle preparations to antigen was significantly reduced following incubation of these tissues with compound I suggesting the possibility that, in addition to reducing antigen-induced tension generation, the release of anaphylactic mediators by lung tissue mast cells may have also been reduced.

4. Preliminary in vivo studies with compound I in the anesthetized rabbit indicate that orally administered compound I appears to show some success in diminishing adverse respiratory physiological changes associated with either histamine challenge in the control animal, or antigen challenge in the sensitized rabbit as indicated by the decreased histamine - or antigen-induced lung resistance measurements following compound I administration.

5. Enzyme kinetic analysis of rabbit bronchial smooth muscle cyclic AMP-phosphodiesterase (low $K_m$) indicates that compound I is an apparent competitive inhibitor of this enzyme, and this enzyme inhibition has been suggested to be potentially responsible for the observed physiological changes associated with compound I in the rabbit.
6. Compound I and MIX were both shown to be potent inhibitors of the low $K_m$ cyclic AMP-phosphodiesterase present in human peripheral lung tissue indicating that, should phosphodiesterase inhibition prove to be the mechanism responsible for bronchodilation in the rabbit in response to compound I, a similar response may be expected in human airway smooth muscle.

7. Finally, based on these findings, compound I may have significant therapeutic potential in the treatment of obstructive airway diseases.
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