TAURINE AND THE CARDIAC SARCOLEMMA

by

Leslie Ann Sebring

A Dissertation Submitted to the Faculty of the
COMMITEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1987
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Taurine and the cardiac sarcolemma

Sebring, Leslie Ann, Ph.D.
The University of Arizona, 1987
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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Leslie Ann Sebring entitled Taurine and the Cardiac Sarcolemma and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: Leslie A. Schrag
ACKNOWLEDGEMENTS

This work is dedicated to my grandparents, Frank and Anza Morris, for their loving patience and understanding; to my colleague, Mindy Fuchs, for her steadfast friendship; and to my dissertation director, Dr. Ryan J. Huxtable, for his expert guidance and support during these first toddling steps toward becoming a scientist.
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ABSTRACT

Taurine is by far the most abundant of the sulfur amino acids, levels in the heart exceeding the combined quantities of all others. Taurine exhibits extensive cardiovascular pharmacology, including inotropic and antiarrhythmic properties. Many of the actions of taurine appear to involve a modulation of calcium availability.

The sarcolemma regulates the entry of calcium into the heart. Binding sites on the cardiac sarcolemma provide calcium for contraction and maintain membrane integrity. The effect of taurine on calcium binding to rat heart sarcolemma varies with the buffer. In Tris and the presence of sodium, taurine increases the affinity of the low affinity binding, but decreases the maximal binding of calcium. In the absence of sodium, taurine decreases affinity of the low affinity binding without altering the maximal binding. These effects on low affinity binding, however, are absent in physiological buffers representative of extracellular conditions. In buffers representative of intracellular ionic conditions, taurine increases the high affinity binding of calcium to sarcolemma in a dose-dependent manner. These results suggest that taurine exerts its cardiotonic actions through a modulation of the high affinity calcium binding on the internal aspect of the sarcolemma.

Membrane phospholipids are important calcium-binding molecules in cardiac sarcolemma. Heterogeneous vesicles containing phospholipids in a ratio approximating that of rat heart sarcolemma bind significant quantities of calcium. Taurine increases calcium binding to the artificial liposomes in a
manner similar to that observed for sarcolemma. Taurine also increases calcium binding to homogeneous vesicles of phosphatidylserine, but not phosphatidylinositol, phosphatidylcholine or phosphatidylethanolamine.

Taurine modulation of calcium may not involve a classical protein-ligand interaction, but, instead, a low affinity attraction to sarcolemmal phospholipids. Taurine binds to sarcolemma with low affinity and positive cooperativity at concentrations normally present in the rat heart. Neither β-alanine nor guanidinoethane sulfonate, inhibitors of taurine transport, affect taurine binding. However, hypotaurine and various cations reduce binding.

Heterogeneous phospholipid vesicles also bind taurine with positive cooperativity which was enhanced by the inclusion of cholesterol. Taurine associates with homogeneous vesicles of phosphatidylcholine, phosphatidylserine, or phosphatidylethanolamine. Phosphatidylinositol bind little taurine.

These studies support the hypothesis that taurine exerts its modulation of sarcolemmal function through an interaction with membrane phospholipids.
CHAPTER 1

INTRODUCTION

General Information on Taurine

Taurine (2-aminoethanesulfonate) is a sulfur-containing β-amino acid which does not incorporate into protein (fig. 1). The interest in the function of taurine in the heart stems directly from the high concentration present in cardiac tissue. It is by far the most abundant free amino acid in the heart, comprising in excess of 50% of the total amino acid pool. In the rat heart, for instance, taurine levels exceed 30 umol/g wet weight of tissue; i.e. approximately 35 mM free amino acid (Huxtable, 1976). Indeed, concentrations of the next most abundant amino acid, glutamate, are one-fourth that of taurine (Huxtable et al., 1979). There is an intuitive feeling that a substance present in such abundance must be serving some important functions, but what these functions are has thus far eluded clear definition.

Taurine has long been known as a constituent of living organisms. Tiedemann and Gmelin (1827) first acknowledged the presence of taurine in ox bile, but referred to the isolated compound as bile-asparagine. Within half a century, the nearly ubiquitous distribution of taurine in mammalian tissues, as well as tissues of other vertebrate and invertebrate species, was well documented in extensive surveys by Krukenberg (1881) and von Furth (1903).

In contrast to the relative abundance in animals, taurine has only limited distribution in plants. Taurine has been identified in a number of
marine algae, predominately of the subclass, Rhodophyceae (Ericson and Carlson, 1954). Taurine is absent in algae of fresh water origin (Ericson and Carlson, 1954; Fowden, 1951). A few genera of fungi contain taurine (Fuerst and Wagner, 1957; Kelly and Weed, 1965). In higher plants, taurine has only been demonstrated in pollen from five dicotyledonous genera (Marquardt and Vogg, 1952). The relative absence of taurine in plants prompted Karas et al. (1964) to suggest the use of taurine as a chromatographic reference in analyses of plant extracts.

Occurance in Humans

Significant concentrations of taurine are present in human tissues. Levels in myocardium are intermediate between those reported for adult human liver (0.3 umole/g wet weight) and adult human spleen (11.4 umole/g wet weight). Huxtable and Bressler (1974), for instance, found approximately 6 umole/gm wet weight in the hearts of patients dying from noncardiac causes.

Taurine concentrations may differ markedly during disease. Loss of taurine from heart tissue follows the acute stress of myocardial infarction or coronary bypass surgery (Cooper and Lombardini, 1981; Lombardini and Cooper, 1982). Conversely, the taurine content of the human heart may increase several fold in response to chronic stress leading to congestive heart failure (Huxtable and Bressler, 1974).

Moderate levels of taurine are also reported in human brain, with relatively higher concentrations present in the frontal and occipital lobes (Okumura et al., 1960). Disease states confined to the central nervous system apparently do not affect taurine levels. Brain specimens from patients with brain tumors, tuberous sclerosis (Okumura et al., 1960), Lowe's syndrome, or
Fig. 1. Structure of Taurine

\[ \text{H}_2\text{N-CH}_2\text{-CH}_2\text{-SO}_3\text{H} \]

TAURINE
unspecified mental retardation (Zachman, Tocci, and Nyhan, 1966) do not have taurine levels which differ significantly from the levels in normal human brains. The taurine content of the cerebrospinal fluid of 18 mental defectives, including two patients with Down's syndrome, were similarly not altered. However, in disorders in which there is a coupling between the central nervous system and motor activity, there appears to be a change in taurine concentration. Huxtable et al. (1979) have reported an increased level of taurine in all examined brain regions of two patients with Friedreich's ataxia. Furthermore, the biopsied sites of maximal seizure activity in epileptic patients are characterized by low concentrations of taurine (Van Gelder et al., 1972).

Taurine levels are substantially lower in human body fluids. The concentration of taurine in plasma or serum from normal adults and from children after the first few weeks of life has been reported to range from 25 to 150 umol/liter (Jacobsen and Smith, 1968). The considerable range of the normal serum concentrations have lead to suggestions that spuriously high levels were obtained due to lysis of taurine-rich leukocytes and platelets. A method in which the plasma is separated from formed elements of the blood by rapid dialysis, thus preventing cell lysis, has found taurine present in concentrations much less than 20 umol/liter (Frame, 1958; Soupart, 1962). The level of taurine in plasma does not appear to be influenced by sex (Ackerman and Kheim, 1964), pregnancy (Christensen et al., 1957), or treatment with cortisol (Zinneman, Seal, and Doe, 1963), estrogen, or progesterone (Zinneman, Seal, and Doe, 1967).

In urine from adults, taurine is the most abundant ninhydrin-positive compound after glycine (Jacobsen and Smith, 1968). The amount of taurine
excreted daily varies markedly from one individual to another and in the same individual from day to day. Acceptable values for daily taurine excretion encompass a wide range from 220 to greater than 2600 umoles. The renal clearance differs as widely, ranging from 0.2 to 20 ml/min (Traver and Schmidt, 1942; Cusworth and Dent, 1960; Ling, 1957). These variations in taurine clearance, however, may result in part from uncertainty about the true levels of taurine in plasma. Calculations of the percentage of filtered taurine, which is reabsorbed in the renal tubules, give an average value of 94.2%, indicating inefficient reabsorption.

Increased excretion of taurine in the urine of adults occurs in numerous conditions linked to general aminoaciduria. An increase in urinary taurine output has been observed following accidental exposure to radiation (Cavalieri et al., 1960), traumatic events such as burns (Nardi, 1954), surgical procedures (Turner and Blum, 1964), and acute infections (Berry, 1960), as well as muscle disorders such as myotonia dystrophy (Blahd, Bloom, Drell, 1955).

**Regulation of Tissue Taurine Content**

In spite of the fluctuating urinary excretion and low plasma levels of taurine, tissue contents of the amino acid are quite stable over time. This suggests that constancy of concentration is physiologically important. Taurine levels in the tissues can conceivably be maintained by metabolism, biosynthesis, transport, or a combination of these processes.

**Taurine Metabolism**

Taurine is the sulfur-containing end product of methionine metabolism. The sulfur is present in the form of a sulfonate group and further oxidation
does not occur in mammals. As a result, taurine is biochemically quite stable and the metabolic degradation, extremely slow. One pathway of significance in mammals is conjugation of taurine with cholic acid to form the bile salt, taurocholate (discussed below). Bile acid formation, however, is restricted to hepatic tissues.

Conversion of taurine to isethionic acid is the only metabolic process utilizing taurine as a substrate that has been proposed to exist in the heart (Read and Welty, 1962). One major obstacle in studying this metabolite has been the lack of a sensitive assay. Original detection of the isethionic acid in the heart depended upon crystallization and subsequent weighing of the salt (Read and Welty, 1962). A substance claimed to be isethionic acid was identified as a taurine metabolite in dog heart on the basis of paper chromatographic analysis (Read and Welty, 1962). The isolated compound had the same R\textsubscript{F} of isethionic acid and migrated with carrier on paper electrophoresis. More recently, however, a sensitive technique involving gas chromatography failed to detect isethionic acid in rat or dog heart, and only 2 μg/g tissue was found in rat brain (Applegarth et al., 1977). These results provide evidence against catabolism as a regulatory mechanism in maintaining taurine concentrations in the tissues.

Taurine Biosynthesis

Taurine biosynthesis is a complex problem in that several routes have been established or postulated, the distribution of which vary from species to species, and organ to organ. Some of the enzymes involved in the biosynthesis have now been purified to homogeneity or characterized.
In many mammalian tissues, taurine biosynthesis begins with the conversion of methionine to cysteine. Two divergent pathways subsequent to cysteine are postulated (fig. 2). The most extensively studied pathway is the one from cysteine via cysteine sulfinic acid and hypotaurine to taurine. The enzymes involved in catalyzing the first two reactions are cysteine dioxygenase (EC 1:13:11:20) and cysteine sulfinic acid decarboxylase (EC 4:1:1:12).

Alternatively, cysteine sulfinic acid may first oxidize to cysteic acid, followed by a decarboxylation to taurine. The balance of evidence indicates that the decarboxylation of cysteine sulfinic acid and cysteic acid are catalyzed by the same enzyme, and requires pyridoxine (B₆) as a cofactor (Jacobsen et al., 1964; Guiin-Rain et al., 1975). This enzyme has been purified from bovine brain to homogeneity and is distinctly different, however, from that responsible for L-glutamic acid decarboxylase activity (Wu, 1982). Under similar assay conditions, the affinity of cysteic acid for the decarboxylase enzyme is one-eighth to one-half that of cysteine sulfinic acid in all tissues examined (Yamaguchi et al., 1973). The progression from cysteine sulfinic acid to hypotaurine, therefore, is probably the preferred pathway. This pathway, however, has not been detected in the heart.

A third route of taurine biosynthesis involving the intermediates cysteamine and hypotaurine has been clearly demonstrated to occur in the hearts of certain species. The metabolic origin of the cysteamine, however, is unclear. Currently, the only known pathway by which cysteine may be converted to cysteamine involves the hydrolysis of coenzyme A (Cavallini et al., 1978). If this pathway were the major source of cysteamine, taurine synthesis would be dependent on the turnover of coenzyme A, an unlikely
TAURINE BIOSYNTHESIS

\[
\begin{align*}
\text{Cysteine} & : \quad \text{H}_2\text{N}-\text{CH}-\text{CH}_2-\text{SH} \\
\quad & \quad \quad \quad \downarrow \\
\text{Cysteine Sulfinic Acid} & : \quad \text{H}_2\text{N}-\text{CH}-\text{CH}_2-\text{SO}_2\text{H} \\
\quad & \quad \quad \quad \downarrow \\
\text{Cysteic Acid} & : \quad \text{H}_2\text{N}-\text{CH}-\text{CH}_2-\text{SO}_3\text{H} \\
\quad & \quad \quad \quad \downarrow \\
\text{Hypotaurine} & : \quad \text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{SO}_2\text{H} \\
\quad & \quad \quad \quad \downarrow \\
\text{Taurine} & : \quad \text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{SO}_3\text{H}
\end{align*}
\]

Fig. 2. Taurine Biosynthesis
possibility. Indeed, Novelli and coworkers (1954) find that little, if any, coenzyme A is hydrolyzed to produce cysteamine. Alternatively, Huxtable (1978) has proposed that pantothenic acid, independent of its function in coenzyme A synthesis, acts as a carrier group for cysteine in the production of cysteamine.

In contrast to what little is known of the origin of cysteamine, the oxidation of cysteamine to hypotaurine is well understood. Cysteamine dioxygenase activity is present in extracts of liver and heart muscle of rat, rabbit, mice and guinea pig (Huxtable and Bressler, 1976). Moreover, the enzyme has been partially purified and the enzyme kinetics measured (Cavallini et al., 1965). The possible role of the cysteamine-taurine pathway in human tissues is of particular importance because no significant cysteine sulfinic acid decarboxylase activity is present apart from minimal activity in human brain. Cysteamine, however, has been shown to be a precursor of taurine in man, but studies of human tissues in vitro are lacking.

The final step in both the cysteine sulfinic acid and cysteamine pathways is the conversion of hypotaurine to taurine, catalyzed by the enzyme hypotaurine oxidase. Injection of [35S]hypotaurine results in rapid appearance of [35S]taurine in both mice and rats suggesting that hypotaurine oxidation is not the rate limiting step in the biosynthesis of taurine (Fellman et al., 1980; Sturman and Fellman, 1982). An enzymatic conversion of hypotaurine to taurine in mouse brain slices has also been reported, apparently requiring NAD⁺ as a cofactor (Oja and Kontro, 1981). No information is available concerning the conversion of hypotaurine to taurine in the heart, beyond the fact that it occurs.
Taurine Transport

Circulating taurine derived from biosynthetically active tissue or from a dietary source is taken up by various tissues throughout the body. Intracellular concentrations of taurine often exceed that of the surrounding fluid by several orders of magnitude. Since there is no evidence that intracellular taurine is sequestered, the maintainence of such taurine concentration gradients indicates the existence of mechanisms for active taurine transport. A number of reports have considered the characteristics of taurine uptake in tumor cells because of the extremely high concentration gradients existing across the cell membrane. The concentration of taurine in the cell water of growing Ehrlich tumor ascites cell and HeLa cells, for instance, are approximately 1,000 and 7,000 times greater, respectively, than the concentration in the surrounding medium (Christensen et al., 1964; Piez and Eagle, 1958). Moreover, Kromphardt (1963) has shown that the uptake of taurine into tumor cells is reduced by anoxia and by 2, 4-dinitrophenol, inhibitors of mitochondrial metabolism.

In Ehrlich tumor cells, taurine uptake was inhibited by structurally related $\beta$-amino acids such as $\beta$-alanine and hypotaurine (fig. 3) but was unaffected by $\alpha$-amino acids (Christensen, 1964). At least six transport sites apparently mediate the uptake of the $\beta$-amino acids, but these sites are not completely specific. For example, $\beta$-alanine, the carboxylic analog of taurine, is taken up at either the alanine-preferring sites or those designated for the $\alpha$-amino acids. Taurine, on the other hand, is excluded entirely from the sites for $\alpha$-amino acid transport, but had three times the affinity of $\beta$-alanine for the $\beta$-amino acid site.

These observations have been confirmed and extended to a number of
Fig. 3. Taurine Analogs
mammalian tissues. Detailed kinetic analysis of taurine transport into the isolated perfused rat heart, for instance, indicates the presence of a saturable, high affinity transport process, with a $K_m$ of 45 $\mu$M and a maximum velocity of 32 nmole/g dry weight/min (Chubb and Huxtable, 1978). The uptake is competitively inhibited by the structural analogs of taurine, such as $\beta$-alanine, hypotaurine, and GES.

Modification of Cardiac Taurine Concentration

Cardiac taurine concentrations have proven difficult to manipulate under a range of experimental conditions. Rats fed a diet deficient in pyridoxine or fasted for prolonged periods maintain unaltered levels of taurine in the heart, but show severely reduced excretion (Sturman, 1973). Supplementation of the diet with taurine also has no effect on cardiac taurine concentrations; the additional taurine is simply excreted in the urine and feces (Awapara, 1956). However, Huxtable and Chubb (1978) have demonstrated a significant stimulation of taurine influx in the isolated rat heart in the presence of $\beta$-adrenergic agonists. This may explain the elevations in taurine noted in prolonged states of stress, such as congestive heart failure.

The invariant levels of taurine may be related to the extremely slow rate of taurine turnover in the heart, the half-life being on the order of 15 days (Sturman et al., 1976). In vivo injection of $[^{35}\text{S}]$taurine into humans indicate two general pools of taurine. One pool is relatively small and turns over rapidly. Liver, pancreas, and kidney are examples of organs belonging to this group. A second, much larger pool turns over at a much slower rate. Skeletal muscle, brain and heart belong to this latter group. These results are consistent with those of other in vivo studies involving rats and rabbits.
(Kromphardt, 1963; Awapara, 1957; Guinnebault et al., 1956). More detailed studies by Huxtable and Lippincott (1982) in which rat pups obtained radio-labeled taurine in utero, during nursing, and after weaning have different conclusions. Under these conditions, the viscera exchange taurine at a faster rate than taurine is eliminated from the body with no indication of biphasic kinetics of elimination. The half-life of elimination of taurine from the heart after weaning in these pups was approximately six days, compared to eleven days from the whole body.

Physiological Functions of Taurine

"Our knowledge of the function and fate of the various sulfur-containing compounds is incomplete and can be likened to an unfinished mosaic" (Dziewiakowski, 1968). This quotation from a review of sulfur amino acids some eighteen years ago is particularly applicable to taurine even today. Although taurine probably existed before the origin of life, little is known about its physiological importance. Taurine appears to have several unrelated functions: osmoregulation, bile salt formation, and neuromodulation.

Osmoregulation

One of the earliest recognized phylogenetic functions of taurine was that of an osmotic agent in bacteria (Measures, 1975), molluscs (Hoyaux et al., 1976) and fish (Forster and Goldstein, 1979; Fugelli, 1980). As the osmotic environment alters, the concentrations of taurine in the cell changes to maintain osmotic equilibrium. Taurine may fulfill a similar function in mammals. In the brains of hypernatremic mice, the major change in osmolarity is provided by taurine (Thurston et al., 1980). In hyponatremic mice, as plasma
sodium falls from 200 to 142 mEq/l, the concentration of taurine in the heart falls from 35.1 to 22.2 umole/g wet weight (Welty et al., 1976). In hypernatremic mice, as cardiac water content decreased from 310 to 263 g/100 g dry weight, taurine concentration increased from 34 to 44 umole/g wet weight. No other free amino acid increased in concentration, although several decreased (Thurston et al., 1981).

Bile Salt Formation

The participation of taurine in bile salt formation is its most firmly established physiological function. Conjugation of bile acids with taurine and glycine occurs in most vertebrates above the selachians (Jacobsen and Smith, 1968). The ratio of taurine to glycine conjugates is species specific and influenced by diet (Doisy et al., 1956; Haselwood, 1962). Generally, the ratio is lower in herbivores than carnivores. In man, taurocholic acids are present in a ratio of about 1:3 compared to those of glycine conjugates.

Bile contains three lipid constituents of limited solubility: bile salts (of calcium), phosphatidylcholine and cholesterol. Normal micelle formation of these constituents and thus, cholesterol solubility, is critically dependent on the concentration of conjugated bile acids such as taurocholate. If taurine (or glycine) is unavailable for bile acid conjugation, a decreased metabolism of cholesterol would result in elevated in serum levels. This hypothesis was examined by the work of Portmann and Mann (1955) in which hypercholesterolemia and atherosclerosis were experimentally induced by feeding primates a diet high in cholesterol and low in sulfur amino acids. The hypercholesterolemia was easily reversed by adding methionine, cysteine, or taurine to the diet. More recently, taurine has been added to the diet of man and hyperchole-
estolemic rabbits in an attempt to lower serum cholesterol levels by increasing bile salt conjugation (Truswell et al., 1965 Burns and Self, 1969). In these studies, dietary taurine increased the taurine to glycine ratio but was ineffective in lowering serum cholesterol. The results suggest that cholesterol metabolism is not affected by manipulations of bile acid conjugation in man.

Neuromodulation

Taurine is anticonvulsant in a large variety of genetic and experimental epilepsies. Taurine reduces seizures of the genetically seizure-susceptible rat (Huxtable and Laird, 1978), the photosensitive baboon (Wada et al., 1975) and the vestibularly sensitive mouse (Iwata at al., 1979). Taurine is also anticonvulsant in the experimental models of epilepsy of penicillin-induced seizures (Durelli et al., 1976), cobalt-induced seizures (VanGelder, 1972), the hypoxic rat (Sanberg and Willow, 1980) and pentylene tetrazole-induced seizures (Izumi et al., 1974). Human epileptics resistant to other forms of treatment have also been effectively treated with taurine (Huxtable, 1981, for review).

The problem of whether taurine acts as a neurotransmitter or neuromodulator in the mammalian central nervous system has long been a matter of discussion. Taurine fulfills many of the criteria required of a neurotransmitter. For example, taurine has an inhibitory effect on the rate of neuronal firing when applied iontophoretically in the vicinity of the neuron (Curtis and Watkins, 1960; Haas and Hosli, 1973). A high affinity, active uptake mechanism selective for taurine is present in neuronal tissue (Lahdesmaki and Oja, 1973; Schmidt et al., 1975). Moreover, a high-potassium evoked release from neurons has been demonstrated (Katz et al., 1969).
Other studies support the idea that taurine may act as a modulator of neuronal activity. Kuriyama et al. (1978), for instance, report an inhibitory effect of taurine on the high-potassium-evoked release of acetylcholine and norepinephrine from excitable tissues. Taurine also reduces active calcium transport in a number of neuronal tissues (Kuriyama et al., 1983). In any case, it is apparent that taurine has a prominent inhibitory action on neurons in the central nervous system.

Pharmacology of Taurine in the Heart

Although a physiological function in the heart remains undefined, taurine exhibits an extensive cardiovascular pharmacology. The various effects may be summarized briefly: inotropic actions (both intrinsic actions and potentiation of digitalis inotropy), antiarrhythmogenic actions, and membrane stabilizing effects. Many of the actions of taurine appear to involve a modulation of ion fluxes in general, and calcium availability in particular.

Inotropy

Taurine produces a biphasic effect on cardiac inotropy. At low extracellular calcium concentrations, taurine is positively inotropic (Dietrich and Diacano, 1971; Dolara et al., 1978). In contrast, taurine decreases inotropy when calcium levels are raised above physiological concentrations. In the spontaneously beating bullfrog heart, for example, at 2.8 mM calcium, taurine produced a maximum 10% increase in tension, whereas at 0.5 mM calcium taurine produced a maximum increase of 78% in tension (Read and Jaqua, 1980). Furthermore, at calcium concentrations above 1.8 mM, taurine produced a short-lived decrease in tension development followed an increase.
Khatter et al. (1981) report similar effects on contractility in the isolated guinea pig heart. Here, taurine produced an increase in inotropy, the extent of which was inversely proportional to the calcium concentration over the range of 0.5 to 2.0 mM calcium. In a more detailed study, Franconi et al. (1982), using guinea pig ventricular strips, found that 4 mM taurine was positively inotropic at 0.5 mM calcium, whereas at 1.8 mM calcium taurine concentrations had to be increased to 20 mM for a positively inotropic effect. At 2.7 mM calcium, the action of taurine was reversed, and a negative inotropy was observed. In this manner, the maximum tension that can be developed in the presence of taurine is less, but the response at low calcium concentrations is magnified.

Taurine also increases the inotropic response to digitalis glycosides. In the rat and guinea pig heart, ouabain and taurine had additive effects at low calcium concentrations (Dietrich and Diacano, 1971). In guinea pig auricles, taurine increased the contractile response to strophanthin-K (Guidotti et al., 1969). Similar effects were reported by Dolara et al. (1973) and in dogs by Chazov et al. (1974). The lowering of extracellular potassium increases the inotropic responses to ouabain and the taurine augmentation of the ouabain effect (Iwata and Fujimoto, 1976).

Digitalis Induced Arrhythmias

The effect of taurine on the arrhythmias of digitalis toxicity remains controversial. Read and Welty (1963) found that in dogs intravenous administration of taurine prevented arrhythmias caused by acute or chronic dosing with digitalis. Acutely, intravenous digoxin was administered until the onset of premature ventricular contractions. Subsequent administration of
intravenous taurine abolished the toxic arrhythmias. Digitoxin was also given by mouth for four weeks until evidence of electrocardiographic toxicity developed. Taurine administered in substantial doses (5 - 10 mmole/kg) again effectively abolished the ventricular arrhythmias. Chazov et al. (1974) report similar antagonism of strophanthin-K toxicity by taurine in isolated guinea pig atria. Taurine also inhibited the reduction in isolated atria inotropy that normally accompanies ouabain toxicity. Fujimoto (1977) suggested that the antiarrhythmic of taurine may be attributed to a taurine prevention of intracellular potassium deficit produced by toxic levels of the digitalis glycosides.

Others have not been able to show such clear-cut anti-arrhythmic effects of taurine. For example, intravenous taurine (0.01 - 4.0 mmole/kg) failed to convert the sustained ventricular tachycardia in cats made toxic by the administration of deslanoside (Hinton et al., 1975). Pretreatment with taurine also failed to protect. In fact, in one-half of the animals, taurine appeared to exacerbate the arrhythmias. This arrhythmogenic action of taurine may be related to the very high doses of taurine employed. Three mmole/kg is equivalent to a 26 g dose for a 70 kg human. Similarly, dogs given digoxin until the appearance of sustained ventricular tachycardia were unprotected by pretreatment or subsequent treatment with taurine. Such differing effects of taurine as an antiarrhythmic agent suggest an inability to disrupt sustained arrhythmias such as ventricular tachycardia, although taurine may by helpful in the prevention of the prevention of isolated ectopic firing.

Calcium Turnover

Cardiac energetics and contractility are controlled by the movements of
calcium. Langer (1974) has resolved the calcium washout kinetics of the rabbit ventricle into four phasic components plus an inexchangeable pool. The components obtained by analysis of a washout pool may or may not correspond to physical pools of calcium. Exchange of phases 0 and 1 are perfusion limited (the faster the heart is perfused, the shorter the half lives of these components) and have been equated with intravascular and interstitial calcium, respectively. This rapidly exchangeable calcium is necessary for contraction, in that calcium-free perfusion leads to a fall in contractility with a half-life for tension decrease of 45 to 72 seconds. Phases 2 and 3 are equated with the intracellular pools of calcium that are necessary, but insufficient for contraction.

A direct effect of taurine on calcium movements was first reported by Dolara et al. (1973). Isolated guinea pig hearts were perfused with a physiological buffer in the presence or absence of 8 mM taurine for 15 min. The hearts were then perfused for 1 min with a calcium-free buffer. The hearts exposed to taurine released more calcium during the calcium-free washout, maintained contractility longer, and had more calcium retained at the end of the washout. The investigators contribute these observations to a taurine-induced enhancement of calcium affinity of some intracellular structure for calcium.

Chubb and Huxtable (1978) observed essentially the same phenomenon in rat heart perfused with radiolabeled calcium for 15 min, and washed out for 2 min with calcium-free solution. Hearts exposed to 8 mM taurine effluxed more radioactivity, and had more radioactivity remaining in them at the end of the washout period, indicating that taurine had increased the uptake rate of radioactive calcium. These findings provide a direct demonstration that
taurine affects a slow-exchanging kinetic pool of calcium.

Calcium Binding

For taurine to be exerting such a marked effect on calcium exchange in the heart, it must be modifying the interaction of calcium with some cell structure. It was at one time thought that direct complex formation occurred between taurine and calcium. However, Dolara and coworkers (1978) using natural abundance of \([^{13}\text{C}]\) NMR, found the stability constant for complex formation to be low. They estimated that approximately 8% of the free calcium in the heart is bound to taurine. Irving and coworkers (1982) refined this approach by using \([^{13}\text{C}]\)-enriched taurine, and calculated association constants even lower than those of Dolara et al. (1978). As a result, they estimated that only slightly over 1% of the free calcium in the heart is associated with taurine. This eliminates a direct complexation between taurine and calcium as the mechanism of the marked calcium-modulatory actions of taurine.

Taurine and Sarcolemma The sarcolemmal actions of taurine form a crucial area for current research on taurine. Cardiac sarcolemma shares many features with cell membranes in general and excitable membranes in particular. "Sarcolemma" generally designates the 7.5 to 9 nm lipid bilayer membrane that encloses the cytoplasm of the muscle cell, but a more complete definition includes the specialized surface coat on the external aspect of the membrane (McNutt, 1975). This external network, termed the glycocalyx, is composed primarily of mucopolysaccharides, having a total width from 20 to 60 nm. The glycocalyx is rich in acidic phospholipids and sialic acid residues; both contribute to the negative surface charge at physiological pH. These residues
bind calcium and may be a partial source of extracellular calcium involved in excitation-contraction. The acidic phospholipids of the sarcolemmal lipid bilayer have also been postulated to represent a possible pool for the calcium involved in excitation-contraction coupling (Philipson et al., 1980).

One of the functions of the sarcolemmal membrane is to provide a barrier between the high extracellular concentration of calcium (of the order of $10^{-3}$ M) and the low intracellular concentration ($10^{-9}$ M - $10^{-5}$ M). The sarcolemma also serves to regulate the entry of calcium into the cell in response to various stimuli, such as membrane depolarization. Calcium binding to the sarcolemma has been analyzed in terms of two types of sites, differing in affinity and capacity. Typically, Bers and Langer (1979) found a relatively small number of sites with high affinity ($K_m$ 0.02 mM) and a much larger number with low affinity ($K_m$ 1.2 mM).

Literature concerning the interaction of taurine and the binding of calcium to the sarcolemma appears confused and often contradictory. There are reports that taurine increases, decreases, or is without effect on calcium binding. For example, in a Tris buffer of high sodium and low potassium concentrations, taurine has been reported to increase the total calcium bound to the low affinity sites without affecting the high affinity binding (Chovan et al., 1979). When the incubation buffer was replaced with a Tris buffer containing low sodium and high potassium concentrations, taurine potentiated calcium binding to both low and high affinity sites (Chovan et al., 1980). Opposite effects have been reported with bicarbonate buffers (Azari and Huxtable, 1980; Franconi et al., 1982). When sarcolemma was incubated in a Krebs-bicarbonate buffer, taurine reduced calcium binding (Azari and Huxtable, 1980). This buffer contained 2.5 mM calcium, a concentration well within the
range of the low affinity binding sites. A further variable modifying the response to taurine may be the presence or absence of ATP. Welty and Welty (1981) have measured an increase in high affinity calcium binding with the addition of taurine and ATP at 1 uM calcium, but not at 100 uM calcium. Other studies, however, have demonstrated taurine enhancement of calcium binding at 100 uM calcium in the absence of ATP.

It may be possible to rationalize these apparent discrepancies in terms of variables, such as calcium concentration, buffer composition, or the presence of ATP, that may interact with taurine to modify calcium binding. From the discussion above, it is clear that much work is required to clarify this area.

**Taurine and the Sarcoplasmic Reticulum** Under physiological conditions, the sarcoplasmic reticulum in the heart has a major function in lowering cytosolic calcium concentrations, resulting in myofibril relaxation. Whether the sarcoplasmic reticulum is also involved in releasing the sequestered calcium to raise cytosolic levels for contraction remains controversial.

There is one report that taurine in concentrations as low as 1 uM increased calcium binding to sarcoplasmic reticulum of guinea pig heart (Dolara et al., 1976). Numerous subsequent studies, however, fail to confirm an action of taurine on calcium binding. Taurine has no effect on calcium binding or calcium–magnesium ATPase activity of sarcoplasmic reticulum from spontaneously hypertensive or Wistar rats (Chubb and Huxtable, 1976). This enzyme is responsible for the rapid uptake of calcium into the sarcoplasmic reticulum during cardiac relaxation. Others have found no effect on the sarcoplasmic reticulum of guinea pig heart (Khatter et al., 1981), dog heart (Entman et al., 1977), or hamster heart (Welty and Welty, 1981).
Taurine and Mitochondria  Mitochondria are unlikely to be involved in the regulation of cardiac calcium under physiological conditions because the affinity for mitochondrial calcium binding is far higher than normal cytosolic calcium concentration. Thus, it has been calculated that mitochondria remove less than 1% of the calcium in the mammalian heart that needs to be sequestered for relaxation to occur.

Dolara et al. (1973), studying liver mitochondria, found that taurine increased ATP-dependent calcium uptake. However, an action of taurine was only seen at 10 mM calcium, a concentration far higher than would be obtained in a normal cell. Taurine has been reported to increase calcium binding and ATPase activity of guinea pig cardiac mitochondria at calcium concentrations as low as 0.1 mM (Khatter et al., 1981). On the other hand, calcium binding to cardiac mitochondria from random-bred hamsters was unaffected by taurine, whereas taurine depressed calcium binding to mitochondria from cardiomyopathic hamsters (Welty and Welty, 1981). Calcium ATPase activity was depressed in both strains.

It appear that under certain circumstances the handling of calcium by mitochondria can be modified by taurine. How much this may be responsible for the change in the kinetics of calcium exchange in the whole heart is unclear.

Calcium Paradox

Calcium paradox refers to the phenomenon that occurs when a heart undergoes calcium-free perfusion, followed by re-exposure to physiological concentrations of calcium. Removal of calcium from the tissue perfusate produces, within minutes, ultrastructural aberrations and marked changes in
membrane permeabilities. Reperfusion of the heart with solutions containing physiological concentrations of calcium levels results in further cellular derangements, termed the "calcium paradox" by Zimmerman and colleagues (1966).

Two prominent structural changes are associated with the calcium paradox. First, the sarcolemmal membrane is disrupted in regions of the intercalated discs with subsequent loss of intercellular cohesions (Muir, 1867). Electron microscopic studies of the effects of calcium depletion indicate a specific separation of the glycocalyx into an external lamina and an underlying surface coat attached to the lipid bilayer. These changes have been correlated with the subsequent loss of membrane bilayer integrity (Yates, 1975). The sarcolemma loses the capacity to regulate calcium entry and prevent potassium leakage as well as nonspecific leakage of large intracellular components such as creatine phosphokinase and myoglobin into the perfusate. Re-exposure of the cardiac muscle is associated with a massive influx of calcium and the development of myofibrillar contracture. The extent of the damage that results on reperfusion is a function of the length of time of calcium-free perfusion.

Two calcium pools are involved in producing the calcium paradox: one involving the sialic acid residues in glycocalyx cohesions (Frank et al., 1977), and the other being responsible for the maintenance of the integrity of the phospholipid bilayer (Crevey et al., 1977). Both pools must represent rather high affinity sites for calcium interaction since as little as 50 uM external calcium will maintain the glycocalyx and membrane bilayer structure and function, thus, preventing the calcium paradox phenomenon.
The presence of taurine is also protective against the calcium paradox. Dolara et al. (1978) found that in guinea pig ventricular strips exposed to calcium-free perfusate the presence of taurine during calcium reperfusion enhanced the recovery of contractile force. Kramer et al. (1981) further established that for a given degree of contractile recovery, 10 mM taurine increased the tolerated calcium-free perfusion time by about 1 to 2 minutes. Again, taurine was only needed during the reperfusion phase. Taurine was ineffective, however, in preventing the loss of contractile force following ischemia, produced by a low rate of perfusion of the isolated working heart.

Cardiomyopathy

The cardiomyopathic hamster has a genetic impairment of its ability to regulate calcium entry in the heart. As a result, there is progressive intracellular accumulation of calcium, leading to cellular damage and eventual necrosis. Investigators have postulated that the initial defect involves reduced sarcolemmal calcium binding and thus less calcium availability for contraction (Ma and Bailey, 1979). The resultant decrease in contractility would promote reflex sympathetic overactivity and progression to calcium overload.

Three arbitrary stages have been defined in this progressive model of cardiomyopathy: at 30 days of age, there are no areas of necrosis, at 60 days of age, necrotic lesions have started to appear, and by 120 days, the animal is in an advanced stage of necrosis (Azari et al., 1980). When taurine is given orally at 0.1 M solution ad libitum over the period 30 to 60 days of life, calcium accumulation is markedly decreased (McBroom and Welty, 1977). Detailed studies further showed that the number and severity of necrotic lesions were also decreased by the taurine treatment (Azari et al., 1980). The
effect was apparently specific for calcium, as cardiac magnesium and iron content were only minimally affected.

**Rationale and Objectives**

It is clear from the above discussion that taurine modulates calcium in the heart. An important aspect of these cardiotonic actions of taurine is that there are currently no pharmacological agents used in treatment which exhibit the biphasic actions of taurine. The ability to increase calcium availability for contraction and yet at the same time protect against calcium overload toxicity is unique. An understanding of the biochemistry of this phenomenon is of immense potential importance therapeutically.

Recent investigations have focussed on taurine and the cardiac sarcolemma. Binding sites on the sarcolemma provide the calcium necessary for the maintainance of membrane integrity (Crevey et al., 1977) and excitation-contraction coupling (Bers et al., 1981). However, studies concerning the interaction of taurine upon the binding of calcium to sarcolemma have been contradictory. Moreover, the molecular mechanism mediating the membrane response to taurine has not been elucidated.

In view of the enormous physiological and pharmacological concentrations, the binding of taurine with a high affinity protein receptor on the sarcolemma, distinct from that for amino acid transport, therefore, appears unlikely. One possibility is that taurine may alter sarcolemmal calcium by virtue of an ion-ion interaction with membrane phospholipids. Phosphatidylserine and phosphatidylinositol are important calcium-binding molecules in cardiac sarcolemma (Philipson et al., 1980). Taurine is reported to protect the integrity of the phospholipids, in that taurine reduces membrane lipid per-
oxidation (Alvarez and Storey, 1983) or degradation by phospholipase-C (Huxtable and Bressler, 1973). These observations imply a low affinity attraction of taurine to membranes phospholipids.

The specific questions to be answered by this work are:

1. What are the effects of various buffer conditions on the taurine modulation of calcium binding to the sarcolemma?
2. Are the effects of taurine on sarcolemmal calcium attributable to an interaction with phospholipids?
3. Can a low affinity association between taurine and sarcolemma be demonstrated?
4. If taurine binding to sarcolemma is present, does it represent an interaction with membrane phospholipids?
CHAPTER 2

METHODS AND MATERIALS

Animals

Male Sprague-Dawley rats, weighing 200-350 g were obtained from either Hilltop Lab Animals, Chatsworth, California or from the Division of Animal Resources at the University of Arizona. Animals were housed at the University of Arizona in a room maintained at 25°C, and under artificial illumination from 6 a.m. to 6 p.m. The animals were allowed free access to food and water.

Chemicals

$^{45}$Ca]Calcium chloride and $^{14}$C]mannitol was obtained from Amersham, Arlington Heights, Illinois. $^{3}$H]Taurine was prepared by tritium exchange (New England Nuclear, Boston, Massachusetts) and purified through ion exchange chromatography (Hruska et al., 1977). Guanidinoethanesulfonate (GES) was synthesized from taurine by the method of Huxtable et al. (1979) and contained 0.033% taurine. Hypotaurine was purchased from the Calbiochem Company, Los Angeles, California and contained 0.33% taurine. Phospholipids (dissolved in ChCl$_3$) were obtained from the Sigma Chemical Company, St. Louis, Missouri. All other compounds were obtained from commercial sources in the purest available form.
Sarcolemmal Preparation

Cardiac sarcolemma was prepared by modification of the procedure by Singh et al. (1975). Male Sprague-Dawley rats were decapitated, hearts quickly removed, and perfused with 10 ml of ice-cold 10 mM Tris HCl (pH 7.4), containing 1 mM EDTA. Hearts were then homogenized with 10 volumes of Tris-EDTA. The homogenates were filtered through coarse gauze and centrifuged at 1,000 x g for 10 min. The sediment was suspended in 10 mM Tris, washed for 30 min, then centrifuged at 1,000 x g for 10 min. This process was carried out three times, with the pellet being retained. The final sediment was extracted with 10 mM Tris, containing 0.4 M LiBr, for 45 min and centrifuged at 1,000 x g for 3 min. The pellet was again washed three times with 10 mM Tris and suspended in the appropriate buffer at a final protein concentration of 2 to 8 mg/ml. Final protein concentration was determined according to the method of Lowry et al. (1951) with bovine serum albumin serving as a standard. All isolation procedures were carried out at 4°C.

Electron Microscopy of Sarcolemma

Isolated sarcolemma was prepared for electron microscopy as described by Frank et al. (1977). The primary fixative was glutaraldehyde (2%) buffered with 0.1 M cacodylate buffer (pH 7.1). Postfixation in 1% osmium tetroxide was preceded by a brief rinse in 0.1 M cacodylate buffer. The tissue was dehydrated in ethanol and then embedded in Spurr's Resin. After curing, the resin was cut with glass knives on an ultramicrotome.

Prior to embedding, some of the tissues were labeled with colloidal iron hydroxide, a specific histochemical probe for sialic acid. Colloidal iron hydroxide was prepared according to a modification of the procedures of Gasic
et al. (1963). Five ml of 0.5 mM FeCl₃ were added to a rapidly boiling distilled water (60 ml). After cooling, 10 ml of glacial acetic acid were added to the colloidal iron hydroxide solution and the pH adjusted to below 1.8. The tissues were exposed for 40 min to the colloidal iron hydroxide, followed by a 10 min wash with several changes of 12% acetic acid. Postfixation and embedding of these tissues were as described as above. All electron microscopic photography and tissue fixation, with the exception of the colloidal iron hydroxide preparation, were graciously performed by Mary Hendrix, Ph.D., Department of Anatomy, University of Arizona.

ATPase Activity of Sarcolemma

ATPase activities of the isolated sarcolemma were determined in the presence of various cations. All incubations were done at 37° C. The basic medium was Tris HCl, pH 7.2 (20 mM). ATP (4 mM) was used as the Tris salt, prepared by passage of the ATP through an AG50 (H⁺)ion exchange column, followed by adjustment to pH 7.2 with Tris base. Ion concentrations used for the different assays were NaCl, 140 mM; KCl, 14 mM; MgSO₄, 3 mM and CaCl₂, 2 mM. The incubation medium was preincubated for 1 min and the reaction was started by adding sarcolemma (0.2 – 0.5 mg protein/ml). Total volume of incubation was 2 ml and length of incubation was 10 min. The incubation was terminated by the addition of 5% trichloroacetic acid, followed by rapid centrifugation. Inorganic phosphate content of the supernatant was measured according to the method of Huxtable and Bressler (1973).
Phospholipid Vesicle Preparation

Phospholipid vesicles were prepared according to Philipson et al. (1980). Purified phospholipid (1-4 mg, dissolved in CHCl₃) were dried under a stream of dry nitrogen and resuspended in 1 ml of 5 mM Tris HCl (pH 7.4). The suspension was sonicated for 1 hr at ambient temperature in a bath-type sonicator (Bransonic Model 220). Heterogeneous vesicles included phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine (18:19:2:1) in the approximate proportions previously reported for rat heart sarcolemma (Tibbets et al., 1981). Cholesterol (50%) was included in some of the heterogeneous vesicles.

Calcium Binding to Sarcolemma

Calcium binding was measured with an equilibrium dialysis system (Spectrum Medical Industries, Inc., Los Angeles, California). The dialysis apparatus consisted of 10 teflon half cells, 1.0 ml volume for each half cell. The dialyzing membrane was Spectropor, with a molecular weight cutoff of 12,000 to 14,000. Half cells were loaded with buffer containing either ⁴⁵Ca²⁺ or suspended sarcolemma. After 45 min, 20 ul samples were taken from each cell and ⁴⁵Ca²⁺ content determined by liquid scintillation counting (Beckman LS 250). Sarcolemmal-bound calcium was calculated as the calcium present in the protein compartment less that present in the dialysate. The Tris-HCl buffer concentration was 10 mM, pH 7.4. Simple bicarbonate buffers contained only 140 mM NaCl and sodium bicarbonate at the concentrations indicated in each experiment. Unless otherwise specified, Krebs-Henseleit buffer had the following composition: NaCl, 120 mM; Glucose, 5.5 mM; KCl, 4.7 mM; MgSO₄·7H₂O, 1.2 mM; KH₂PO₄, 1.2 mM and NaHCO₃, 25 mM. The bicarbonate
and Krebs-Henseleit buffers were bubbled with 100% CO₂ only until pH 7.4 was achieved. Less than a 2% change in buffer pH was measured following a 45 min dialysis interval in the closed dialysis system. Final free Ca⁡²⁺ in the high affinity binding studies was titrated by the addition of EDTA (Fabiato and Fabiato, 1979). Apparent stability constants were derived from the absolute constants listed by Fabiato and Fabiato (1979), and adjusted for pH 7.4. The Ca⁡²⁺-ATP stability constant was taken from O'Sullivan and Perrin (1964). Unless otherwise indicated, the magnesium concentration in each Krebs-Henseleit buffer in the high affinity binding studies was increased to 5 mM to overcome the chelation by ATP (when present) of Ca⁡²⁺.

**Calcium Binding to Isolated Phospholipids**

Calcium binding to isolated phospholipids was assayed by a biphasic technique similar to that described by Feinstein (1967). An aqueous solution (0.5 ml) containing 140 mM NaCl; 10 mM Tris HCl (pH 7.4); and 0.125 - 2.25 mM CaCl₂ was added to an organic solution (1.0 ml) consisting of a 2:1 mixture of chloroform-methanol (v/v). ⁴⁵Ca²⁺ was initially present in the aqueous solution at a specific activity of approximately 0.5 uCi/umole of CaCl₂. The incubation was initiated by the addition of phospholipid (1 mg, dissolved in 10 ul chloroform) and the mixture vortexed for 1 min. Clear separation of the chloroform and aqueous phases was obtained after standing for 5 min. Both phases were assayed for radioactivity by liquid scintillation counting. Taurine (10 mM) was included in some of the samples.
Calcium Binding to Phospholipid Vesicles

The high affinity binding of calcium to phospholipid vesicles (0.5 to 2 mg) was measured by a dialysis procedure similar to that described for sarcolemma. The dialyzing buffer has the following composition: Tris-HCl, 20 mM (pH 7.4); NaCl, 5 mM; KCl, 110 mM; MgSO₄·7H₂O, 1 mM. When the concentration of phospholipid was high (2 mg/ml), the inaccessible space within vesicles was estimated in a duplicate incubation containing an excess of EDTA.

Calcium Efflux from Sarcolemma

Passive calcium efflux from the sarcolemma was measured following sonication and a 1 hr incubation with ⁴⁵Ca²⁺ in Tris or Krebs-Henseleit buffers. The sarcolemma was then diluted into a medium containing EDTA (1 mM). Sarcolemma-retained calcium was calculated as the calcium present in the protein compartment less that present in the dialysate at each time point.

Taurine Binding to Sarcolemma

Taurine binding to sarcolemma was measured by centrifugation. Each incubation contained 20 mM Tris-HCl (pH 7.4) and 0.1 to 150 mM [³H]taurine (0.3 - 0.7 uCi/ml). The incubation medium (total volume, 1.0 ml) was placed in 1.5 ml polypropylene microfuge tubes and maintained at 22°C. Binding was initiated by the addition of 0.2 ml aliquots of sarcolemma (1 to 2 mg of protein). After 60 min, the incubation was terminated by brief centrifugation (3 min) in a Beckman Microfuge B. The supernatent was discarded and the inner aspect of the microfuge tubes was carefully wiped dry. The pellet was resuspended in 1 ml of deionized water to facilitate dissolution in scintillation cocktail. The tips of the microfuge tubes containing the resuspended pellet were placed in scin-
scintillation vials and the radioactivity was extracted from the tissue for at least 24 hr into scintillation cocktail before determination by liquid scintillation spectrometry (Beckman LS 1500). In appropriate incubations, unlabeled taurine or various other compounds were added to the incubation medium 30 min. following the addition of the membranes.

Taurine binding to isolated sarcolemma was also measured by standard filtration methods. Incubation conditions were identical to those described above except that the incubation was terminated by rapid suction filtration of the sample onto Millipore filters (type HA WP, 0.45 uM pore size). Each filter was washed twice with 1.0 ml ice-cold Tris buffer (10 mM, pH 7.4), allowing 1 min between subsequent washings. Radioactivity was extracted from the filters for 24 hr into scintillation cocktail before determination by liquid scintillation spectrometry.

The specific binding of taurine was defined as the total binding less the nondisplacable binding measured in the presence of 250 mM unlabeled taurine. The limited solubility of taurine in solution precluded the addition of unlabeled taurine in excess of 250 mM. Whenever incubations contained 10 mM radiolabeled taurine or greater, the calculation of specific binding included an appropriate correction. For example, the addition of 250 mM unlabeled taurine to incubations containing 50 mM radiolabeled taurine would only displace some 83% of the specifically-bound taurine from the tissue. In this instance, the actual specific binding is 1.2 times the displaced radiolabeled taurine. In general, the correction factor was determined by the equation: (250 + x)/250, where x represents the concentration of radiolabeled taurine.
Taurine Binding to Phospholipid Vesicles

Phospholipid vesicles were incubated with radiolabeled taurine in a procedure identical to that described for sarcolemma. Binding was terminated by centrifugation of the sample in microfiltration tubes fitted with nitrocellulose filters, 0.2 uM pore size (BAS). Following a 15 min centrifugation (2000 x g) in a fixed angle rotor (Sorvall), the filters were removed and radioactivity was determined as above. The low affinity nature of the taurine binding to phospholipids did not permit washing the filters following centrifugation. The background binding of taurine to the filters in the absence of phospholipid accounted for 30% of the average radioactivity on the filter.

Stability of Phospholipid Vesicles

Phosphatidylserine (1 mg) was dried under a stream of dry nitrogen and resuspended in 0.5 ml of 100 mM Tris HCl (pH 7.4), containing 10 mM NaCl and 0.5 uCi [14C]mannitol in a trace amount. The suspension was sonicated for 5 min at ambient temperature in a bathtype sonicator (Bransonic Model 220). Bulk phase mannitol was separated from that incorporated into the sonicated vesicles by passage through a Sephadex G-50 column (1.5 x 25 cm), eluted with tracer-free Tris-sodium buffer. The collected membrane dispersion was immediately dialyzed against an equivalent Tris-sodium buffer and the absence and presence of calcium (1 - 5 mM) or taurine (40 mM). The amount of the mannitol isotope appearing in the dialysate during the first hour of dialysis was taken to represent the permeability of the phospholipid vesicles at the various calcium concentrations.
Phospholipid Content of Sarcolemma

Phospholipids were extracted from sarcolemma according to the method of Bligh and Dyer (1959). Resuspended sarcolemma (1 ml, from 1 g of tissue) was homogenized in CHCl₃:methanol:water (2:2:1 ml). Following a 5 min separation, the aqueous layer was discarded and the remaining organic layer evaporated under a stream of dry nitrogen. The phospholipids were hydrolyzed by briefly boiling the sample (1 min) in 0.8 ml sulfuric acid (10 N), followed by 2 drops of concentrated nitric acid and 1 ml of water (Philipson and Bers, 1980). The lipid portion of the cleaved phospholipids was extracted into 1 ml of CHCl₃ and the residual aqueous phase assayed for the presence of inorganic phosphate (Huxtable and Bressler, 1973).

Data Analysis

Low affinity binding constants of the sarcolemmal calcium binding and the binding constants of the sarcolemmal and phospholipid taurine binding were estimated by nonlinear regression analysis, Statistical Package for the Social Sciences, McGraw-Hill Book Company. Binding curves were fitted to the equation: $B = \frac{B_{max}(L)^n}{(K_d + (L)^n)}$, where $B$ represents the amount of taurine binding at a given free concentration of the ligand ($L$), $B_{max}$ represents maximal binding, $K_d$ represents the binding dissociation constant, and $n$ represents the number of cooperative sites (Hill coefficient). The Hill coefficients determined for the low affinity binding of calcium did not differ from unity; thus, this term in the binding equation was deleted from those calculations.

Calcium efflux rate constants were estimated by nonlinear regression analysis fitted to the equation: $y = Ae^{-kt}$, where $y$ represents the amount of
sarcolemmal calcium remaining, $A$ represents the initial sarcolemmal calcium, $k$ is the rate constant, and $t$ is the time elapsed since dilution of the sarcolemma into an EDTA buffer. The half-life was calculated as $t_{1/2} = 0.693/k$.

Significant differences were determined by Analysis of Variance, 'F' test, followed by the paired or unpaired Student's 't'-test, with a probability of $p < 0.05$ taken as indicating a significant difference. The paired Student's 't'-test was used to determine differences in calcium binding in the absence and presence of taurine within the same buffer since each sarcolemma or phospholipid preparation served for both control and treated measurements.
CHAPTER 3

RESULTS

Sarcolemmal Characterization

Isolated sarcolemmal fractions were assayed for purity using several marker enzymes (table 1). Compared to the original heart homogenate, the preparation was found to have a 10-fold enrichment of the magnesium-dependent, Na⁺/K⁺ ATPase activity, inhibitable by ouabain (10⁻⁵ M). Calcium (2 mM) also decreased the activity of the Na⁺/K⁺ ATPase. Similar calcium inhibition of this enzyme has been previously described for isolated dog heart sarcolemma (Sulakle and Dhalla, 1971). No significant inhibition of calcium ATPase activity was noted in the presence of sodium azide (5 mM), a mitochondrial inhibitor (data not shown). Less than 1% of the total protein in the initial homogenate was recovered in the sarcolemmal fraction.

The membranes isolated in this manner were also examined by electron microscopy. The micrographs depict closed vesicles of varying shape (fig. 4c and 4d). No cristate structures were apparent by electron microscopy that would indicate mitochondrial contamination.

For the purposes of electron microscopy, some of the membranes were isolated in the presence of calcium (0.1 mM), in an attempt to maintain the glycocalyx that adheres to the outer surface of the sarcolemma. The glycocalyx can be visualized with the aid of colloidal iron hydroxide, a specific
Table 1. ATPase Activities and Yield of Sarcolemma

<table>
<thead>
<tr>
<th></th>
<th>Homogenate</th>
<th>Sarcolemma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg\textsuperscript{2+}-dependent, Na\textsuperscript{+}/K\textsuperscript{+} ATPase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.56 ± 0.38</td>
<td>5.85 ± 0.57\textsuperscript{a}</td>
</tr>
<tr>
<td>Control + Ouabain</td>
<td>ND</td>
<td>3.71 ± 0.59\textsuperscript{b}</td>
</tr>
<tr>
<td>Control + Ca\textsuperscript{2+}</td>
<td>ND</td>
<td>ND\textsuperscript{b}</td>
</tr>
<tr>
<td>Yield (mg protein)</td>
<td>1020 ± 219</td>
<td>7.9 ± 2.2\textsuperscript{c}</td>
</tr>
</tbody>
</table>

ND = None detectable above ATPase activity in the presence of Mg\textsuperscript{2+} alone.
\textsuperscript{a} p < 0.05 compared to homogenate control activity.
\textsuperscript{b} p < 0.05 compared to sarcolemmal control activity.
\textsuperscript{c} p < 0.01 compared to homogenate yield.

Enzyme activities are umole Pi/mg protein/h. The Mg\textsuperscript{2+}-dependent, Na\textsuperscript{+}/K\textsuperscript{+} ATPase represents the ATPase activity in the presence of magnesium, sodium, and potassium less that measured in the presence of magnesium alone. Results expressed are the mean ± SEM of 4 separate sarcolemmal preparations.
Fig. 4a, 4b, 4c, 4d. Electron Microscopy of Isolated Sarcolemma — Sarcolemma isolated in the presence (4a, 4b) or absence (4c, 4d) of 0.1 mM calcium chloride were stained with colloidal iron hydroxide. Magnification = 42,000x.
histochemical probe for sialic acid residues that are abundant in the outer lamina.

With electron microscopic examination, a dense band of colloidal iron staining was present in the preparations containing calcium, but absent in those isolated with calcium-free conditions (fig. 4b and 4d, respectively). Membranes prepared with calcium, however, were grossly contaminated with other intracellular structures (fig. 4a and 4b). Intact nuclei and mitochondrial cristae were discernable, thus the isolation was inadequate. The sarcolemma used in the remainder of this dissertation was prepared in the absence of calcium.

Taurine and Calcium Binding to Sarcolemma

Low Affinity Binding of Calcium to Sarcolemma

Sarcolemmal calcium binding was studied by an equilibrium dialysis technique. With this method, the binding of calcium to sarcolemma achieved equilibrium within 30 min (fig. 5). Following equilibration, the binding remained constant for at least 2 hr.

The low affinity binding of calcium (0.5 - 7 mM) to sarcolemma incubated in Tris buffer is shown in Figure 6. The addition of 140 mM Na\(^+\) to Tris drastically decreased the affinity for calcium, but increased the binding capacity of the sarcolemma (fig. 6; table 2). This effect is not simply a result of changing the ionic strength of the buffer as choline chloride was less potent that NaCl in reducing sarcolemmal calcium binding. For example, at a calcium concentration of 0.5 mM, calcium binding in the presence of 140 mM choline chloride was 23.3 ± 2.9 nmole/mg protein, compared to 14.8 ± 1.8 nmole/mg protein in the presence of 140 mM NaCl.
Fig. 5. Equilibration and Stability of Calcium Binding to Sarcolemma in Tris Buffer — Calcium concentration was 0.3 mM. The results represent the mean ± SEM of calcium binding to three independent sarcolemmal preparations studied from 5 to 300 min.
Fig. 6. Effect of Taurine on the Low Affinity Binding of Calcium to Sarcolemma in Tris Buffer — In the upper pair of curves, sodium chloride was omitted from the Tris incubation. In the lower pair of curves, the incubation medium included 140 mM NaCl. Each pair of curves represents the mean ± SEM of 5 individual binding curves determined from independent sarcolemmal preparations studied over the entire calcium range in the absence (open circles) or presence (closed circles) of taurine (10 mM). *p < 0.05 compared to calcium binding at an equivalent free calcium concentration in the absence of taurine, paired Student's 't' test.
Table 2. Effect of Taurine on Low Affinity Binding of Calcium to Rat Heart Sarcolemma in Tris Buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Kd (mM)</th>
<th>Bmax (n mole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris^a</td>
<td>0.203 ± 0.045</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>Tris + Taurine</td>
<td>0.289 ± 0.039^b</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Tris + NaCl</td>
<td>4.60 ± 1.00^c</td>
<td>155 ± 12^c</td>
</tr>
<tr>
<td>Tris + NaCl + Taurine</td>
<td>1.90 ± 0.28^c,d</td>
<td>106 ± 8^d</td>
</tr>
</tbody>
</table>

^a p < 0.01, analysis of Variance 'F' test comparing Kd and Bmax constants in all Tris buffers.

^b p < 0.05 compared to Tris, paired Student's 't' test.

^c p < 0.05 compared to Tris, unpaired Student's 't' test.

^d p < 0.05 compared to Tris + NaCl, paired Student's 't' test.

Binding constants were estimated by nonlinear regression analysis. Results are expressed as mean ± SEM of 4-5 separate sarcolemmal preparations. Binding in the presence or absence of taurine for each buffer type was measured in the same preparations. Calcium concentration range was 0.5 to 7 mM (see figure 6). Sodium concentration when present was 140 mM. Taurine concentration when present was 10 mM.
The binding of calcium (0.5 - 7 mM) to sarcolemma in sodium bicarbonate and Krebs-Henseleit buffers is shown in figures 7 and 8, respectively. Increasing the concentration of bicarbonate from 10 to 50 mM did not significantly alter affinity, but progressively decreased maximal calcium binding (fig. 7; table 3). In Krebs-Henseleit buffer (145 mM Na\(^+\)) the maximal binding capacity was similar to that measured in a simple buffer of equivalent bicarbonate concentration (fig. 8; table 3).

Taurine and Low Affinity Binding of Calcium

The effects of taurine on the calcium binding to sarcolemma vary with the buffer composition. In Tris, in the absence of sodium, taurine (10 mM) decreased the affinity of calcium for the sarcolemma without affecting the maximal binding capacity (fig. 6; table 2). With the addition of 140 mM NaCl to the Tris, taurine increased the calcium binding affinity, but decreased the binding capacity. Thus, in Tris buffer, the effects of taurine were biphasic with the direction of change being modulated by sodium.

When the sarcolemma was incubated in bicarbonate buffer, however, no significant effect of taurine on the low affinity binding of calcium was found at the three bicarbonate concentrations tested (fig. 7; table 3). Taurine was also without effect on this low affinity calcium binding to sarcolemma in the physiological Krebs-Henseleit buffer (fig. 8; table 3). Taurine also did not alter the binding to sarcolemma incubated in Krebs-Henseleit at 37\(^\circ\)C (fig. 9).

The differing actions of taurine on the low affinity calcium binding to sarcolemma incubated in Tris and bicarbonate-containing buffers prompted us to examine the properties of these vesicles more closely. The effects of A23187, a calcium ionophore, and lanthanum, a calcium antagonist, showed that sarco-
Fig. 7. Effect of Taurine on the Low Affinity Binding of Calcium to Sarcolemma in Bicarbonate Buffers. In the upper pair of curves, the bicarbonate concentration was 10 mM; the middle pair, 25 mM; and the lower pair, 50 mM. Sodium concentration in the incubations was 140 mM. Each curve represents the mean ± SEM of 4 to 5 individual binding curves determined from independent sarcomemmal preparations studied over the entire calcium range in the absence (open circles) or presence (closed circles) of taurine (10 mM).
Fig. 8. Effect of Taurine on the Low Affinity Binding of Calcium to Sarcolemma in Krebs-Henseleit Buffer — The curve represents the mean ± SEM of 5 individual binding curves determined from independent sarcolemma preparations studied over the entire calcium range in the absence (open circles) or presence (closed circles) of taurine (10 mM).
Table 3. Effect of Taurine on Low Affinity Binding of Calcium to Rat Heart Sarcolemma in Bicarbonate-Containing Buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Kd (mM)</th>
<th>Bmax (nmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate 10 mM</td>
<td>2.42 ± 0.29</td>
<td>164 ± 23</td>
</tr>
<tr>
<td>Bicarbonate 10 mM + Taurine</td>
<td>2.37 ± 0.44</td>
<td>161 ± 34</td>
</tr>
<tr>
<td>Bicarbonate 25 mM</td>
<td>2.84 ± 1.07</td>
<td>106 ± 17</td>
</tr>
<tr>
<td>Bicarbonate 25 mM + Taurine</td>
<td>2.11 ± 0.77</td>
<td>92 ± 12$^b$</td>
</tr>
<tr>
<td>Bicarbonate 50 mM</td>
<td>2.34 ± 1.13</td>
<td>74 ±17$^b$</td>
</tr>
<tr>
<td>Bicarbonate 50 mM + Taurine</td>
<td>3.78 ± 1.06</td>
<td>93 ± 25</td>
</tr>
<tr>
<td>Krebs-Henseleit</td>
<td>3.94 ± 1.43</td>
<td>92 ± 17</td>
</tr>
<tr>
<td>Krebs-Henseleit</td>
<td>4.59 ± 1.61</td>
<td>102 ± 21</td>
</tr>
</tbody>
</table>

$^a p < 0.05$, Analysis of Variance 'F' test comparing Kd and Bmax binding constants in all bicarbonate buffers. Binding constants for Krebs-Henseleit buffers were analyzed separately and not found to be significantly different.

$^b 0 < 0.05$ compared to bicarbonate 19 mM, unpaired Student's 't' test.

Binding constants were estimated by nonlinear regression analysis. Results are expressed as mean ± SEM of 4–5 separate sarcolemmal preparations. However, binding in the presence or absence of taurine for each buffer was measured in the same preparations. Calcium concentration range was 0.5 to 7 mM (see figures 7 and 8). Taurine concentration when present was 10 mM.
Fig. 9. Effect of Taurine on the Low Affinity Binding of Calcium to Sarcolemma Incubated at 37°C — The curve represents the mean ± SEM of 4 individual binding curves determined from independent sarcolemmal preparations studied over the entire calcium range in the absence (open circles) or presence (closed circles) of taurine (10 mM).
lemmal vesicles in Tris buffer were sealed (table 4). In Tris, the addition of A23187 almost doubled calcium sequestration by the sarcolemma. Lanthanum chloride added to calcium-equilibrated sarcolemma in Tris displaced 90% of the bound calcium.

Membranes incubated in Krebs-Henseleit behaved differently. A23187 did not affect the amount of calcium bound to sarcolemma incubated in Krebs-Henseleit buffer, in the presence or absence of ATP (table 4). Lanthanum completely displaced calcium binding in the Krebs-Henseleit incubations, again regardless of the inclusion of ATP. The rate of passive calcium efflux from the sarcolemma upon dilution into a medium containing EDTA was also significantly faster in Krebs-Henseleit than in Tris buffer (fig. 10). Release of calcium from vesicles was the rate-limiting step in diffusion across the semipermeable membrane as calcium and EDTA alone equilibrated more rapidly.

High Affinity Binding of Calcium to Sarcolemma

Scatchard analysis of the sarcolemmal binding of calcium over the concentration range 1 μM - 7 mM in Krebs-Henseleit buffer shows the presence of two types of binding sites, differing both in affinity and capacity (fig. 11). The high affinity calcium sites were examined further at 1, 10 and 100 μM calcium in Krebs-Henseleit buffer of modified compositions. The contribution of calcium binding to the low affinity sites, calculated from the estimated binding constants of Figure 11, were 7%, 9% and 24% at 1, 10, and 100 μM calcium respectively. Thus, even at 100 μM calcium, most (76%) of the calcium is bound to high affinity sites.

Figure 12 shows the high affinity binding of calcium to sarcolemma incubated in three buffers: high Na⁺-low K⁺; low Na⁺-high K⁺; and low Na⁺-low
Table 4. Effect of A23187 and La\textsuperscript{3+} on Calcium Binding to Rat Heart Sarcolemma in Tris and Krebs-Henseleit Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Bound Calcium (nmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris\textsuperscript{a}</td>
<td>3.28 ± 0.37</td>
</tr>
<tr>
<td>Tris + A23187</td>
<td>6.31 ± 0.30\textsuperscript{b}</td>
</tr>
<tr>
<td>Tris + La\textsuperscript{3+}</td>
<td>0.48 ± 0.26\textsuperscript{b}</td>
</tr>
<tr>
<td>Krebs-Henseleit\textsuperscript{a}</td>
<td>0.98 ± 0.17</td>
</tr>
<tr>
<td>Krebs-Henseleit + A23187</td>
<td>0.94 ± 0.17</td>
</tr>
<tr>
<td>Krebs-Henseleit + La\textsuperscript{3+}</td>
<td>ND\textsuperscript{c}</td>
</tr>
<tr>
<td>Krebs-Henseleit + ATP</td>
<td>1.66 ± 0.27</td>
</tr>
<tr>
<td>Krebs-Henseleit + ATP + A23187</td>
<td>1.66 ± 0.22</td>
</tr>
<tr>
<td>Krebs-Henseleit + ATP + La\textsuperscript{3+}</td>
<td>ND\textsuperscript{d}</td>
</tr>
</tbody>
</table>

ND = No detectable binding.

\textsuperscript{a}p < 0.01, Analysis of Variance 'F' test, Tris and Krebs-Henseleit buffers analyzed separately.

\textsuperscript{b}p < 0.01 compared to Tris, unpaired Student's 't' test.

\textsuperscript{c}p < 0.01 compared to Krebs-Henseleit, unpaired Student's 't' test.

\textsuperscript{d}p < 0.01 compared to Krebs-Henseleit + ATP, unpaired Student's 't' test.

Calcium concentration was 0.01 mM. A23187 was added to incubation medium from a stock in DMSO before the addition of calcium. Final concentration of A23187 was 0.1 mM and of DMSO was 1%. DMSO alone was without effect (data not shown). La\textsuperscript{3+} (0.05 mM) was added to the incubation medium after equilibration with calcium, and the medium allowed to re-equilibrate. ATP when present was 1 mM. Results are expressed as the mean ± SEM of 4-5 separate sarcolemmal preparations.
Fig. 10. Calcium Efflux from Sarcolemma in Tris and Krebs–Henseleit Buffers — Semilogarithmic plot of the remaining sarcolemmal calcium against elapsed time after initial dilution into a dialyzing medium containing EDTA (1 mM). Final calcium concentration was 0.25 mM. The half-life in Tris buffer (closed circles) was 19.0±2.3 min, in Krebs–Henseleit buffer (open circles), 13.8±0.2 min, and in the absence of sarcolemma (open squares; some points off scale), 3.4±0.1 min. Each life was significantly different from the others *p < 0.05, Analysis of Variance, 'F' test and Student's 't' test).
Fig. 11. Scatchard Analysis of the Binding of Calcium to Sarcolemma in Krebs-Henseleit Buffer -- Magnesium concentration was 1.2 mM. Each point represents the mean of 4-5 individual determinations from independent sarcolemmal preparations. Binding constants calculated from the illustrated data were: low affinity - $K_d 3.94$ mM; $B_{max} 92$ nmole/mg protein; high affinity - $K_d 0.03$ mM; $B_{max} 9.4$ nmole/mg protein.
K+ Krebs-Henseleit, in which Na+ was limited to the contribution by the sodium bicarbonate of the buffer. Lowering the sodium concentration increased calcium binding. Raising the potassium concentration antagonized this effect. The addition of ATP also enhanced calcium binding to the high affinity sites in high or low Na+ Krebs-Henseleit buffer (fig. 12, 13).

Taurine and High Affinity Binding of Calcium

The action of taurine on the high affinity binding of calcium also varies with the incubation conditions. Taurine decreased the high affinity calcium binding to sarcolemma incubated in a high Na+-low K+ Krebs-Henseleit buffer (fig. 12). When the buffer was replaced with a modified Krebs-Henseleit buffer containing either low Na+-high K+ or low Na+-low K+ concentrations, the effect of taurine was reversed and an enhancement of calcium binding occurred. When ATP was included in the Krebs-Henseleit incubation, taurine increased calcium binding, regardless of the sodium concentration (fig. 13). Similar increases of the high affinity binding by taurine could also be demonstrated in a simple Tris buffer of low sodium-high potassium content (fig. 14). In each case, the greatest differences were noted at the lowest calcium concentration (1 uM) tested and no significant change were measured at 100 uM calcium. The effect of taurine on the high affinity binding of calcium was clearly dose-dependent (fig. 15).
Fig. 12. Effect of Taurine on the High Affinity Calcium Binding to Sarcolemma incubated in Krebs-Henseleit Buffer — The buffer has the following composition: A = high sodium (145 mM)-low potassium (4.7 mM); B = low sodium (4.7 mM)-high potassium (145 mM); and C = low sodium (25 mM)-low potassium (4.7 mM). The results express the mean ± SEM of 3 to 7 independent sarcolemmal preparations studied in the absence (open bars) or the presence (closed bars) of taurine (10 mM). *p < 0.05 compared to the absence of taurine, paired Student's 't' test.
Fig. 13. Effect of Taurine on the High Affinity Calcium Binding to Sarcolemma Incubated Krebs-Henseleit Buffer and ATP — The buffers had the following composition: A — high sodium (145 mM)—low potassium (4.7 mM) and ATP (1 mM); and B — low sodium (25 mM)—low potassium (4.7 mM) and ATP (1 mM). The results express the mean ± SEM of 4 to 9 independent sarolemmal preparations studied in the absence (open bars) or presence (closed bars) of taurine (10 mM). *p < 0.05 compared to the absence of taurine, paired Student's 't' test.
Fig. 14. Dose-dependent Effect of Taurine on High Affinity Calcium Binding to Sarcolemma — The incubation buffer was Krebs-Henseleit buffer including 1 mM ATP. Calcium concentration was 10⁻³ M. Sodium concentration was 25 mM. Taurine concentration in the incubations was 0, 10, 20, or 40 mM. Results are expressed as the mean ± SEM of 5 independent sarcolemmal preparations. *p < 0.05 compared to control, paired Student's 't'test. **p < 0.01 compared to control, paired Student's 't'test.
Fig. 15. Effect of Taurine on the High Affinity Calcium Binding to Sarcolemma Incubated in Tris Buffer — The buffer contained low sodium (5 mM)–high potassium (110 mM) and magnesium (1 mM). Each value represents the mean ± SEM determined from 4 to 5 independent sarcolemmal preparations in the absence (open bars) or presence (closed bars) of taurine (20 mM). *p < 0.05 compared to the absence of taurine, paired Student's 't' test.
Taurine and Calcium Binding to Isolated Phospholipids

The interaction of taurine with calcium binding to isolated phospholipids was measured with a biphasic distribution technique. In the absence of phospholipid, an insignificant amount of $^{45}\text{Ca}^{2+}$ was taken into the chloroform (data not shown). The effects of calcium concentration ($0.0625 - 1.125 \text{ mM}$) on calcium taken into the chloroform phase by phosphatidylserine or phosphatidylinositol is illustrated in Figure 16. The binding to the lipids was saturable, with maximal binding (687 and 872 nmole calcium/mg phospholipid, respectively) representing an approximate calcium to phospholipid molar ratio of 1:2. Phosphatidylcholine and phosphatidylethanolamine, under similar conditions, bound less than 1 nmole calcium/mg phospholipid at each concentration examined (data not shown). Taurine (10 mM), in the aqueous phase, did not significantly alter the binding of calcium to isolated phospholipids.

Taurine and Low Affinity Calcium Binding to Phospholipid Vesicles

Scatchard analysis of the binding of calcium to heterogeneous phospholipid vesicles over the concentration range of $1 \text{ uM} - 5 \text{ mM}$ shows the presence of two types of binding sites, differing in affinity and capacity (fig. 17). Taurine effects on low affinity calcium binding to vesicles of phospholipids were similar to that observed for isolated sarcolemma. In Tris buffer and the absence of sodium, taurine (10 mM) slightly, but significantly, reduced low affinity binding of calcium ($0.025 - 1.0 \text{ mM}$) to phosphatidylserine vesicles (fig. 18). The low affinity calcium binding to phospholipid vesicles in the presence of sodium (140 mM) could not be determined because of vesicle disaggregation.
Fig. 16. Effect of Taurine on Calcium Binding to Isolated Phospholipids -- Calcium binding (0.0625 – 1.125) was measured in the absence (open circles) or presence (closed circles) of taurine (10 mm) to phosphatidylserine (upper curve) and phosphatidylinositol (lower curve). Estimated of maximal binding were 687 and 872 n mole calcium/mg phospholipid, respectively. Each point represents the mean ±SEM of 4 separate preparations.
Fig. 17. Scatchard Analysis of the Binding of Calcium to Phospholipid Vesicles. Each point represents the mean of 4 separate vesicle preparations. Binding constants calculated from the illustrated data were: low affinity - $K_d$ 0.97 mM; $B_{max}$ 22 nmole/mg lipid; high affinity - $K_d$ 0.02 mM; $B_{max}$ 1.7 nmole/mg lipid.
Fig. 18. Effect of Taurine on the Low Affinity Binding of Calcium to Phosphatidylinerine Vesicles in Tris Buffer — The incubation was in the absence of sodium and the absence (open circles) and presence (closed circles) of taurine (10 mM). The curves represent the mean ± SEM of 4 separate vesicle preparations. *p < 0.05 compared to the absence of taurine, paired Student's 't' test.
Taurine and High Affinity Calcium Binding to Phospholipid Vesicles

The high affinity binding of calcium to phospholipid vesicles was examined in a buffer representing intracellular ionic conditions; that is, of low sodium and high potassium concentrations. Homogeneous vesicles of phosphatidylserine or phosphatidylinositol bound significant quantities of calcium (table 5). Taurine (20 mM) increased calcium binding to vesicles of phosphatidylserine, but not phosphatidylinositol. The predominant phospholipids of the sarcolemma, phosphatidylcholine and phosphatidylethanolamine, bound little calcium in the presence or absence of taurine (table 5). Mixed vesicles of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cholesterol, in a ratio approximating that of rat heart sarcolemma, also bound calcium (fig. 19). Taurine increased the calcium binding to these mixed vesicles in a dose-dependent manner.

Taurine and the Stability of Phosphatidylserine Vesicles

Tracer compounds were incorporated into phospholipid vesicles and efflux measured by dialysis. Figure 20 illustrates the separation of intravesicular \(^{14}C\)mannitol from tracer remaining in the bulk phase by passage through a Sephadex G-50 column, eluted with tracer-free buffer. Less than 4% of the tracer was trapped by the lipid vesicles, which pass freely through the column. Fractions #9 - 12 were pooled and dialyzed against a tracer-free buffer and the absence or presence of calcium (1 - 5 mM) or taurine (40 mM). At calcium concentrations greater than 1 mM, a significant increase in vesicle permeability was indicated by rapid mannitol diffusion (fig. 21). Taurine (40 mM) did not alter the stability of phosphatidylserine vesicles in the absence or presence of calcium (1 - 5 mM).
Table 5. Effect of Taurine on High Affinity Binding of Calcium to Phospholipid Vesicles

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>Taurine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>0.016 ± 0.005</td>
<td>0.020 ± 0.010</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>0.011 ± 0.008</td>
<td>0.006 ± 0.005</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>0.596 ± 0.075</td>
<td>0.823 ± 0.102a</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.292 ± 0.039</td>
<td>0.272 ± 0.038</td>
</tr>
</tbody>
</table>

ND = No detectable binding.

a_p < 0.05 compared to phosphatidylserine control group, paired Student's 't' test.

Results are expressed as mean ± SEM of 4-6 separate phospholipid vesicle preparations. Binding in the presence or absence of taurine (20 mM) for each phospholipid was measured in the same preparations. The buffer composition was low sodium (5 mM)-high potassium (110 mM), magnesium (1 mM), and calcium (10^-6 M).
Fig. 19. Dose-dependent Effect of Taurine on High Affinity Calcium Binding to Phospholipid Vesicles — The vesicles contained phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine (18:19:2:1) and cholesterol (59%). The buffer composition was: low sodium (5 mM)—high potassium (110 mM), magnesium (1 mM), and calcium (10^{-6} M). Each result represents the mean ± SEM of 4 separate preparations. *p < 0.05 compared to control, paired Student's 't' test. **p < 0.01 compared to control, paired Student's 't' test.
Fig. 20. Column Separation of Phospholipid Vesicles Containing $^{14}$C-mannitol. Collection of fractions (0.3 min each) was initiated 2 min after the application of the vesicles to the column. Each fraction was assayed (0.1 ml of 0.8 ml total volume) for radioactivity by liquid scintillation counting. Fractions #8 - 14 corresponded to the position of phosphotidylinerine elution, as measured by UV spectrometric absorbance at 260 nm.
Fig. 21. Effect of Taurine on the Stability of Phosphatidylserine Vesicles -- Diffusion of $[^14C]$mannitol from phosphatidylserine vesicles was measured in the absence (open circles) or presence (closed circles) of taurine (40 mM) and calcium (0 - 5 mM). Each point represent the mean $\pm$ SEM of 4 separate vesicle preparations. *p < 0.05 compared to control in the absence of calcium and taurine.
Taurine Binding to Sarcolemma

Determination of Specific Low Affinity Binding of Taurine

The total and nonspecific binding of taurine (0.1 – 100 mM), as measured by centrifugation techniques, is illustrated in Figure 22. Specific binding was equivalently displaced by either unlabeled taurine (250 mM) or CaCl₂ (250 mM). This concentration of calcium aggregates and precipitates the sarcolemma, thus preventing taurine access to the membrane. Specific binding measured in the presence of either displacer was saturable.

Low Affinity Binding of Taurine to Sarcolemma

The low affinity binding of taurine (0.1 – 100 mM) to sarcolemma, as measured by centrifugation techniques, is illustrated in Figure 23. In the absence of cations, the sarcolemma bound a maximum (Bmax) of 661 nmole taurine/mg protein, with a dissociation constant (Kd) of 19.2 mM taurine. Scatchard analysis of the sarcolemma binding of taurine showed a bell-shaped curve (fig. 23, inset). The Hill plot, however, was linear (fig. 24), with an estimated Hill coefficient of 1.90, indicating positive cooperativity. The phospholipid content of the sarcolemma was 313 (+15) nmole lipid/mg protein (data not shown).

Estimates of taurine binding (1 – 50 mM) to sarcolemma by a rapid filtration method yielded qualitatively and quantitatively similar results (fig. 25). With this assay, the sarcolemma bound a maximum (Bmax) of 510 nmole taurine/mg protein, with a dissociation constant (Kd) of 14.1 mM taurine. The estimated Hill coefficient was 3.26, again indicative of positive cooperativity.
Fig. 22. Total and Nonspecific Low Affinity Binding of Taurine to Sarcolemma -- The upper curve represents the total binding of $[^3]H$taurine (closed circles). In the lower pair of curves, nonspecific $[^3]H$taurine binding was determined in the presence of 250 mM unlabeled taurine (open circles) or 250 mM calcium chloride (closed squares). The results represent the mean ± SEM of binding determined from 8 independent sarcolemmal preparations by centrifugation methods.
Fig. 23. Specific Low Affinity Binding of Taurine to Sarcolemma -- In the absence of cations, the sarcolemma bound a maximum of 661 n mole taurine/mg protein, with a dissociation constant of 19.2 mM and a Hill coefficient of 1.90, indicating positive cooperativity. Scatchard analysis yielded a bell-shaped curve (inset). Each point represents the mean ± SEM of binding determined from 8 independent preparations by centrifugation methods.
Fig. 24. Hill Plot of Taurine Binding to Sarcolemma — The data in Figure 2 were transformed using the binding kinetics described. The low affinity binding had a Hill slope of 1.90 and a correlation coefficient (r) of 0.989.
Fig. 25. Low Affinity Binding of Taurine to Sarcolemma by Filtration Method —
The sarcolemma bound a maximum of 510 nmole taurine/mg protein, with a dissociation constant of 14.1 mM and a Hill coefficient of 3.26, indicating positive cooperativity. Each point represent the mean ± SEM of binding determined from 4 to 8 independent preparations.
Inhibition of Taurine Binding to Sarcolemma by Structural Analogues and Cations

Three structural analogues and several cations were examined for their ability to alter the degree of taurine binding to sarcolemma (Table 6). Each inhibitor was present in an equimolar concentration to that of taurine (50 mM). Hypotaurine, the sulfinate analogue of taurine, was an effective antagonist of taurine binding. Neither GES nor β-alanine, inhibitors of taurine transport in the heart (Huxtable, 1980), affected taurine binding to the sarcolemma. Of the cations tested, calcium was the most potent displacer of taurine. Sodium and potassium produced modest, and nearly equal, inhibition of binding.

Low Affinity Binding of Taurine to Phospholipids

Heterogeneous phospholipid vesicles of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (18:19:2:1) bound taurine in a manner similar to that of sarcolemma (fig. 26). The maximal low affinity binding (Bmax) of these vesicles was 418 nmole taurine/mg phospholipid, or approximately 1 nmole taurine bound per 3 nmol phospholipid. The binding had an estimated dissociation constant (Kd) of 63.7 mM. Positive cooperativity was evident in the linear Hill plot, with a Hill coefficient of 1.52 (fig. 27). The bell-shaped Scatchard analysis resembled that of sarcolemma (fig. 26, inset). The addition of cholesterol (50%) in a relative proportion to that of sarcolemma enhanced the affinity of taurine for the mixed vesicles (Kd = 28.8 mM), but decreased total binding (Bmax = 139) (fig. 28).

The low affinity binding of taurine to homogeneous vesicles of each phospholipid is illustrated in Figure 29. Maximal binding to vesicles of phosphatidylcholine and phosphatidylserine was 1215 and 1362 nmole taurine/mg
phospholipid, respectively. The binding declined sharply after a 1:1 molar ratio (1200 n mole taurine/mg phospholipid) was achieved, suggesting vesicle disaggregation. Maximal taurine binding to phosphatidylethanolamine was one-half that of phosphatidylcholine or phosphatidylserine. The affinity of taurine for the homogeneous vesicles followed the rank order: phosphatidylcholine > phosphatidylethanolamine > phosphatidylserine. Vesicles of phosphatidylinositol bound relatively little taurine.
Table 6. Inhibition of Low Affinity Taurine Binding by Structural Analogues and Cations

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analogue</td>
<td></td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>37 ± 9\textsuperscript{a}</td>
</tr>
<tr>
<td>β-alanine</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>GES</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>Cations</td>
<td></td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>19 ± 13\textsuperscript{a}</td>
</tr>
<tr>
<td>Na\textsuperscript{+}</td>
<td>64 ± 9\textsuperscript{b}</td>
</tr>
<tr>
<td>K\textsuperscript{+}</td>
<td>68 ± 7\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}p < 0.01 compared to control group.

\textsuperscript{b}p < 0.05 compared to control group.

Results are expressed as mean ± SEM of 4-6 separate sarcolemmal preparations. Taurine and inhibitor concentration was 50 mM. Binding in the presence or absence of inhibitors was measured in the same preparations. Statistics were evaluated by analysis of variance 'F' test, followed by Student's 't' test for paired samples.
Fig. 26. Low Affinity Binding of Taurine to Heterogeneous Phospholipid Vesicles

The vesicles prepared from phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (18:19:2:1) bound a maximum of 418 n mole taurine/mg lipid, with a dissociation constant of 63.7 mM, and Hill coefficient of 1.52. Scatchard analysis yielded a bell-shaped curve (inset). Each point represents the mean ± SEM of binding determined from 4 to 7 separate phospholipid preparations.
Fig. 27. Hill Plot of Taurine Binding to Phospholipid Vesicles -- The data in Figure 4 were transformed using the binding kinetics described. The low affinity binding had a Hill slope of 1.52 and a correlation coefficient (r) of 0.999.
Fig. 28. Low Affinity Binding of Taurine to Heterogeneous Phospholipid Vesicles Containing Cholesterol — The vesicles prepared from phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (18:19:2:1) with cholesterol (50%) bound a maximum of 139 n mole taurine/mg lipid, with a dissociation constant of 28.8 mM, and a Hill coefficient of 1.52. Each point represents the mean ± SEM of 4 to 6 separate phospholipid preparations.
Fig. 29. Low Affinity Binding of Taurine to Homogeneous Phospholipid Vesicles — Vesicles of phosphatidylcholine (open squares) bound a maximum of 1215 n mole taurine/mg lipid, with a dissociation constant of 59.9 mM and a Hill coefficient of 1.90. Phosphatidylethanolamine vesicles (closed square) bound a maximum of 736 n mole taurine/mg lipid, with a dissociation constant of 70.1 mM and a Hill coefficient of 1.52. Phosphatidylserine (closed circles) bound a maximum of 1362 n mole taurine/mg lipid, with a dissociation constant of 107 mM and a Hill coefficient of 3.83. Phosphatidylinositol (open circles) bound an insignificant amount of taurine. Each point represents the mean of 3 to 9 separate phospholipid preparations.
CHAPTER 4

DISCUSSION

Taurine modulation of calcium availability in cardiac tissue has been studied extensively, but little is known about the molecular mechanism mediating the physiological and pharmacological actions. Our studies have focused on taurine interaction with the cardiac sarcolemma. This membrane regulates the entry of calcium into the myocyte, and it is the site of action for a vast number of pharmacological and toxicological agents that affect the heart: digitalis, adrenergic agonists and blockers, calcium channel antagonists, local anesthetics, antiarrhythmics, tetrodotoxin, metal ions and others.

Unlike the case with discrete intracellular structures such as mitochondria, it is difficult to get pure preparations of sarcolemma, or even to define what purity is. Depending on the isolation procedure, sarcolemma is torn into fragments or sheets, which may or may not reform vesicles. Furthermore, the final preparation may include varying amounts of other intracellular structures. Thus, the sarcolemmal characteristics observed in detail depend on the isolation procedure used.

The sarcolemma–enriched membrane fraction examined in this dissertation was prepared by a modification of the procedure by Singh et al. (1975). This method was chosen because of the relative speed of the isolation (5 hr) and the moderate purity of the final preparation. The 10-fold purification of the magnesium–dependent, Na⁺/K⁺ ATPase activity is approximately
equal to that reported for sarcolemma isolated by sucrose density gradient fractionation (table 1) (Bers, 1979; Philipson et al., 1980). Attempts to retain the glycocalyx in the membrane fraction were unsuccessful in that calcium present during the isolation lead to gross contamination of the membranes by mitochondrial and nuclear fragments (figs. 4a, 4b). Thus, any interaction of taurine on isolated sarcolemma in this dissertation must be assumed not to involve the glycocalyx.

Taurine and Calcium Binding to Sarcolemma

Many discrepancies have been reported on the modulation by taurine of sarcolemmal binding of calcium. One explanation of such variability may lie in the relative difficulty of assessing calcium binding to biological tissues by standard filtration methods (Philipson et al., 1980). Calcium avidly adheres to filters in the absence of tissue. However, excessive filter washings not only reduce this background binding, but also remove tissue-associated calcium. Moreover, our early attempts indicated that taurine can alter the binding of calcium to filters in the absence of tissue (data not reported in this dissertation). In order to avoid such artefacts, we examined calcium binding to sarcolemma by the novel application of an equilibrium dialysis technique. Using buffers of simple composition, the first portion of our studies attempted to unravel the effects of various components of the incubation medium on the response to taurine.

Initially, our studies were concerned with the low affinity sites; i.e., calcium binding over the calcium concentration range of 0.5 to 7.0 mM. These sites respond to fluctuations in extracellular calcium levels and, under physiological conditions, provide calcium necessary for excitation-contraction...
coupling (Bers et al., 1981). In Tris, taurine decreased calcium affinity in the absence of sodium, but increased the affinity in the presence of sodium (fig. 6, table 2). Thus, taurine reduced the difference in calcium affinity produced by sodium. In addition, a decrease in maximal binding was noted when taurine was included in the Tris-sodium buffer. This biphasic response to taurine in the Tris buffer with sodium, that is, a decrease in maximal binding of calcium but an increase in the affinity of the calcium for the membranes, parallels taurine modulation of inotropy. In cardiac muscle preparations, the maximum tension that can be developed in the presence of taurine is less, but the inotropic response at low calcium concentrations is magnified (Read et al., 1980; Khatter et al., 1981; Franconi at al., 1982). Despite this correlation, taurine did not affect the low affinity binding of calcium in bicarbonate-containing buffers. This was true for both simple bicarbonate buffers and the complex Krebs-Henseleit buffer (figs. 7, 8; table 3). These findings are surprising in view of the fact that the taurine-calcium interaction with other isolated membranes such as brain synaptosomes or various retinal preparations require the presence of bicarbonate (Pasantes-Morales and Gamboa, 1980; Pasantes-Morales et al., 1979).

Our results imply that some fundamental difference exists between the sarcolemma incubated in Tris and sarcolemma incubated in bicarbonate-containing buffers. One such distinction lies in the differing calcium permeabilities of sarcolemma vesicles in Tris and bicarbonate buffers. A23187, a calcium ionophore, almost doubled the calcium-binding capacity of the sarcolemma in Tris (table 4). This suggests that vesicles in Tris are relatively impermeable to calcium, and the addition of the ionophore allows access of calcium to interior binding sites. In Krebs-Henseleit buffer, on the other hand, the ionophore was without effect on calcium binding, indicating that sarcolemma
vesicles incubated in this buffer are permeable to calcium.

These conclusions are consistent with the effects of lanthanum chloride in the two buffers. In Krebs-Henseleit buffer, whether or not ATP was present, lanthanum displaced all bound calcium (table 4); thus, all sites are freely accessible to this impermeant cation. In Tris, lanthanum displaced all but about 10% of the bound calcium, this residual calcium presumably representing a small inaccessible portion transported to the interior of the vesicles.

Similarly, calcium washout from calcium-loaded sarcolemma by EDTA chelation occurred more rapidly in Krebs-Henseleit buffer than in Tris (fig. 10). Thus, the effects of taurine over the low affinity range of calcium binding were only observed in the presence of sealed vesicles. That the modulation by taurine in Tris buffer is sodium-dependent further implies that taurine uptake into the vesicles, a sodium dependent process, may be involved. In the absence of A23187, such sealed vesicles apparently have internal environments low in calcium. In this manner, taurine may be acting on the interior of the sealed vesicles where calcium levels are below the range of the low affinity sites.

This possibility is supported by examination of the interaction of taurine with the high affinity binding of calcium.

The high affinity sites on the sarcolemma have a dissociation constant well below the extracellular calcium concentration. As such, the portion of this pool on the exterior of the sarcolemma as presumably saturated under physiological conditions. On the internal aspect of the sarcolemma, the occupancy of these binding sites fluctuates greatly as intercellular calcium levels from \(10^{-9}\) to \(10^{-5}\)M during the contraction-relaxation cycle. One function of the high affinity calcium pool appears to be structural, in that uM concentrations of extracellular calcium are necessary to maintain membrane integrity, and to
prevent inappropriate ion permeabilities (Crevey et al., 1977). In addition, although there are only a small number of the high affinity binding sites, this pool contributes a relatively large fraction of the calcium for excitation-contraction coupling when external calcium levels, and thus saturation of low affinity sites, is markedly reduced (Philipson and Langer, 1979).

As sarcolemmal vesicles incubated in Krebs-Henseleit buffer are permeable, the intravesicular ionic conditions are the same as the outside. In this case, the concept of vesicular "sidedness" is no longer a concern. For instance, when isolated in Tris, sarcolemma form either "inside-out" or "rightside-out" vesicles (Nayler, 1982). Fractions enriched in closed vesicles of one particular orientation have been used to study the regulatory location of divalent cations and cyclic nucleotides. For sarcolemma incubated in Krebs-Henseleit buffer, however, both aspects of the membrane are equally exposed to the calcium in the incubation, regardless of the original orientation. Thus, by varying the composition of the bicarbonate buffer to mimic intracellular or extracellular conditions, rather than altering the membrane orientation of the vesicles, the site of the calcium modulation on the sarcolemma can be inferred.

Our studies indicate that taurine did indeed alter the calcium binding to the high affinity sites of sarcolemma in Krebs-Henseleit buffer (fig. 12). The direction and degree of modulation were dependent on ionic composition. Taurine decreased the high affinity calcium binding to sarcolemma incubated in a high sodium-low potassium buffer. When the buffer was replaced with one of identical ionic strength, but of low sodium-high potassium content, taurine potentiated calcium binding. Similar potentiation occurred when taurine was included in a low sodium-low potassium buffer. Thus, the direction of the response to taurine was inversely proportional to the sodium concentration,
indicating that it was unrelated to taurine transport.

The presence of ATP is a further variable modifying the response of the high affinity calcium to taurine. When ATP was included in the Krebs-Henseleit buffer, taurine increased calcium binding, regardless of the sodium concentration (fig. 13). The mechanism by which ATP can reverse the effects of taurine in high sodium Krebs-Henseleit buffer is not clear. All calcium associated with the sarcolemma in this buffer was displaced by lanthanum. Thus, no ATP-induced calcium gradient with the permeable vesicles would be measured. Welty and Welty (1981) report a similar enhancement by taurine of the calcium binding to high affinity sites in the presence of ATP. Their findings could not be attributed to a taurine interaction with a sarcolemmal calcium-ATPase, as activity of this enzyme was unchanged. Further examination with non-hydrolyzable ATP analogs might provide insight into this action.

In each Krebs-Henseleit buffer, including those containing ATP, taurine is apparently acting to alter the affinity of high affinity sites rather than the capacity, as the magnitude of the effects was greatest at the lowest calcium concentration \(10^{-6}\text{M}\) and insignificant at the highest calcium concentration \(10^{-4}\text{M}\). A Krebs-Henseleit buffer of low sodium, high potassium and uM calcium is representative of the intracellular rather than extracellular environment. On the other hand a bicarbonate buffer with high sodium, low potassium and mM calcium mimics the ionic concentrations outside of the cell. Thus, the taurine increased calcium binding to sarcolemma incubated in buffers approximating intracellular conditions, but had no effect under extracellular conditions.

That taurine may have an intracellular site of action is not surprising. The heart closely regulates cytosolic taurine levels by a selective transport
mechanism, and intracellular concentrations of this free amino acid are enormous (Huxtable et al., 1980). In the rat heart, for example, levels exceed 30 umole/g wet weight of tissue (Huxtable et al., 1980). Furthermore, under stress conditions such as adrenergic stimulation, cardiac taurine stores are markedly increased (Huxtable and Chubb, 1977). Inotropic concentrations of taurine perfused through the heart also supplement intracellular levels (Iwata and Fujimoto, 1976; Franconi et al., 1982).

In view of this, taurine may be acting both physiologically and pharmacologically to modulate the binding of calcium to the internal aspect of the sarcolemma. The reported actions of taurine in the heart correlate well with the functions attributed to the high affinity binding sites; that is, membrane stabilization, selective protection against detrimental ion permeabilities, and augmentation of the calcium pool involved in excitation-contraction coupling during low calcium perfusion. Nayler (1982) has proposed a similar site of action for cyclic AMP in the heart. Although it remains unclear how the high affinity calcium stores on the cytosolic surface of the sarcolemma may be involved in the regulation of contractility, Nayler has suggested that this pool may regulate slow channel density or availability, thereby ultimately controlling calcium entry in the heart.

**Taurine and Calcium Binding to Phospholipids**

The second portion of our studies was designed to determine whether the increase in the high affinity calcium binding to sarcolemma could be attributed to an interaction of taurine with membrane phospholipids. Acidic phospholipids are the primary calcium-binding moieties of cardiac sarcolemma. Philipson et al. (1980) demonstrated three phospholipids predominate in calcium
binding: phosphatidylinositol, phosphatidylserine, and cardiolipin, representing 7.4%, 5.6%, and 0.6% of total sarcolemmal phospholipid content, respectively. The most abundant phospholipids, phosphatidylcholine and phosphatidylethanolamine, accounting for 39.2% and 29.8% of the total content, apparently have little affinity for calcium. Artificial vesicles prepared from phospholipids in the same proportion as exist in sarcolemma are reported to simulate 80% of the expected calcium binding of intact, isolated sarcolemma.

Taurine was ineffective in altering calcium extraction from an aqueous buffer into chloroform by isolated phospholipids (fig. 16). This biphasic method has been used extensively to examine the actions of local anesthetics on membrane calcium (Feinstein, 1964; Blaustein, 1967). However, taurine is highly lipophobic and would not be expected to enter the organic phase in such an assay.

In contrast, the high affinity binding of calcium to heterogeneous vesicles of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine (18:19:2:1) and cholesterol (59%) in Tris buffer was increased by taurine in a similar manner to that observed for sarcolemma (fig. 19). Taurine also increased the calcium binding to homogeneous vesicles of phosphatidylserine (table 5). The results could not be attributed to an alteration by taurine of vesicle stability, as permeability of the phosphatidylserine liposomes was unaltered (fig. 21). Taurine did not effect calcium binding to vesicles of phosphatidylinositol. Little measureable binding was observed to homogeneous vesicles of phosphatidylcholine or phosphatidylethanolamine. These results support the hypothesis that modulation of sarcolemmal calcium by taurine is mediated by an interaction with membrane phospholipids and, in particular, with phosphatidylserine.
Taurine Binding to Sarcolemma and Phospholipids

The final portion of the studies examined the direct association between taurine and the sarcolemma. Taurine binds to low affinity sites on isolated sarcolemma (figs. 23, 26). Qualitatively and quantitatively similar estimates of binding were obtained with both the methods of centrifugation or rapid filtration. The range of this low affinity binding (1 - 100 mM) corresponds to that observed for cardiac concentrations of taurine in vivo (35 mM in the rat heart) (Huxtable, 1980), as well as for the effective pharmacologic doses (5 to at least 40 mM). The binding is specific for taurine since only the sulfinate analogue, hypotaurine, displaces the amino acid (table 6). GES and -alanine, inhibitors of taurine transport, are without affect. The binding kinetics describe a single, low affinity component (Kd = 19.2 mM) with positive cooperative (Hill coefficient = 1.90) (figs. 23, 24). Scatchard analysis of taurine binding to sarcolemma yields a bell-shaped curve. The binding site is cation-sensitive in that the binding is substantially reduced in the presence of equimolar concentrations of calcium or, to a lesser extent, sodium or potassium.

Our results are consistent with those of Kulakowski et al. (1978) and Hirai (1980) who independently reported a component of taurine binding to cardiac sarcolemma with positive cooperativity. These studies, however, included sodium in the incubation buffer. Such methods did not distinguish between the binding of taurine and intravesicular transport, a sodium-dependent process. Kulakowski et al. (1978), for instance, found two components of taurine binding to isolated sarcolemma: one of low affinity and positive cooperativity that corresponds to our findings, and a noncooperative component of higher affinity that probably represents an uptake process.

The possibility that the low affinity binding of taurine to sarcolemma
may also represent intravesicular transport is unlikely. The active uptake of taurine into myocardium has been extensively characterized. The affinity of the transport site for taurine (1 - 45 uM) is far higher than that observed for low affinity binding (19.2 mM) (Huxtable and Chubb, 1977; Franconi et al., 1980). Moreover, the amino acid uptake requires the cotransport of a cation, such as sodium or potassium (Franconi et al., 1980). The low affinity binding, on the other hand, is exhibited in the absence of any cations and substantially reduced in the presence of sodium or potassium. Traditional inhibitors of taurine transport, GES and -alanine, did not affect the low affinity binding. These differentiations clearly distinguish the sarcolemma binding of taurine from transport.

The studies further demonstrate that heterogeneous liposomes of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (18:19:2:1) bind taurine with positive cooperativity (Hill coefficient = 1.52), again yielding a bell-shaped Scatchard curve (figs. 26, 27). A comparison of total binding (Bmax) to that of sarcolemma, however, is difficult as artificial vesicles prepared in combination with phosphatidylethanolamine often contain multiple lamina (Papahadjopoulos and Miller, 1967). As a result, estimates of maximal binding (one taurine binding per three phospholipids) are probably low since the lipophobic taurine has access only to the outermost lamina. Despite this, the affinity of taurine for the heterogeneous liposomes prepared with or without cholesterol (Kd = 28.8 mM and 63.7 mM, respectively) is remarkably similar to that estimated for sarcolemma (Kd = 19.2 mM) (figs. 26, 28).

These observations support the concept that the interaction of taurine with biological membranes involves phospholipids. Taurine, a zwitterion, bears a
strong structural similarity to the polar head groups of the phospholipids (fig. 30). It is unique among the amino acids in that the basic amino group (pKb = 8.74) is balanced by an extremely acidic sulfonate group (pHa = 1.5, isoelectric point = 5.1) (The Merck Index, 1976). However, at physiological pH (pH 7.4 – 7.5), the residual negative charge on taurine (0.05) is insufficient to chelate calcium. Other drugs with acidic functions, such as phenytoin and phenobarbital, have properties in common with taurine. In the central nervous system, for instance, phenytoin, phenobarbital, and taurine effectively reduce neuronal excitability (for review, Huxtable, 1981). Phenytoin and taurine also repress cardiac hyperexcitability resulting from digitalis toxicity (Bigger and Straus, 1972; Read and Welty, 1963). Blaustein (1967) has attributed the membrane stabilizing properties of phenytoin and phenobarbital to an increase in the binding of calcium to membrane phospholipids.

The moderate acidic nature of the sulfonate function appears to be critical. For example, β-alanine, the carboxylate analogue of taurine, is essentially neutral at physiological pH (pKa = 3.6, pKb = 10.16, isoelectric point = 6.9) (The Merck Index, 1976) and cannot mimic the taurine modulation of calcium (Chovan et al., 1980), nor displace taurine binding from the sarcolemma. Highly acidic amino acids, such as glutamate and aspartate (isolectric point = 3.22 and 2.77, respectively), carry a full negative charge at physiological pH and would compete with the acidic phospholipids for the chelation of calcium (White et al., 1978).

The sulfonate function of taurine would probably associate with the positive charge of the amino group of the phospholipid. In such a case, the net negative charge of the lipid nitrogen should predict the affinity of taurine for each phospholipid. This possibility is supported by examination of the taurine
Fig. 30. Structures of Phospholipids

R = FATTY ACIDS
binding to homogeneous phospholipid vesicles (fig. 29). Our studies find that taurine binds best to vesicles of phosphatidylcholine which contain a strongly basic quaternary nitrogen (Seimiya and Ohki, 1973). Phosphatidylethanolamine, containing only a weakly basic primary amine \( (pK_b = 0.5) \), binds taurine less strongly (Feinstein, 1964). Phosphatidylserine has an additional carboxylate group \( (pK_a = 2.25) \) that reduces the net positive charge on the amino nitrogen \( (pK_b = 9.15) \) (Feinstein, 1964). Phosphatidylinerine binds taurine in a similar manner to phosphatidylethanolamine. Phosphatidylinositol, in which the phosphate is esterified to an inositol sugar, contains no amino group and has little affinity for taurine.

An ion-ion interaction of taurine with the lipid amino group would tend to neutralize the net positive charge of the phospholipid. This, in effect, would increase the net negative charge in the region of the lipid phosphate, thereby enhancing the affinity of the phosphate for cations, such as calcium. Any molecule with sufficient positive charge would be attracted to the lipid phosphate. Sodium or potassium, for example, can substitute for calcium at this site (Feinstein, 1964). The basic amino group of taurine, which carries a partial positive charge, could also interact with the phosphate. In this manner, binding of the first taurine molecule (via the sulfonate function) to the lipid amino group would enhance the attraction of a second taurine (via the taurine amino group) to the lipid phosphate. Indeed, our studies indicate that taurine binds with positive cooperativity to sarcolemma or phospholipid vesicles indicative of a two-site interaction. Furthermore, we find two molecules of taurine bind per sarcolemmal phospholipid when the sarcolemma is incubated in the absence of cations. In spite of these observations, the relative importance of the binding of a second taurine molecule to the lipid phosphate remaine unclear as binding
at this site would be highly susceptible to displacement by cations. However, the ability of taurine to enhance affinity of the lipid phosphate for other cations, such as calcium, may be essential for the taurine modulation of sarcolemmal function.

The results of this final portion of the studies demonstrate a low affinity binding of taurine to cardiac sarcolemma. The binding was qualitatively and quantitatively reproducible in artificial vesicles prepared from pure phospholipids. Many distinctive features of the taurine interaction with sarcolemma, such as cooperative binding, cation sensitivity, and calcium modulation, can be understood in terms of phospholipid biochemistry.

Summary

The work presented in this dissertation has addressed the four questions proposed in the introduction.

1. What are the effects of various buffer conditions on the taurine modulation of calcium binding to the sarcolemma?

This question was asked to establish an accurate hypothesis for taurine induced changes of sarcolemmal calcium content. While taurine altered the low affinity binding of calcium in Tris buffer, the effects were absent in physiological buffer representitive of extracellular fluid compartment. However, incubations with a calcium ionophore and a calcium antagonist indicated that sarcolemmal vesicles incubated in Tris, but not in bicarbonate-containing buffers, were sealed vesicles with internal environments low in calcium. Taurine did increase the affinity of the high affinity calcium binding to sarcolemma in buffers mimicking intracellular conditions.
2. Are the effects of taurine on sarcolemmal calcium attributable to an interaction with phospholipids?

This question proposed a specific site for taurine interaction with the sarcolemma. Taurine increased calcium binding to phospholipid vesicles in a manner similar to that observed for sarcolemma. Taurine also increased calcium binding to homogeneous vesicles of phosphatidylserine, but not to phosphatidylinositol. Other phospholipids bound little calcium, and were unaffected by taurine.

3. Can a low affinity association between taurine and sarcolemma be demonstrated?

Taurine bound to sarcolemma with low affinity and positive cooperativity. The binding occurred at concentrations normally present in the rat heart. The binding was inhibited by hypotaurine, a structural analog, and cations.

4. If taurine binding to sarcolemma is present, does it represent an interaction with membrane phospholipids?

This question poses the hypothesis that has been formulated from the above questions; namely, that taurine exerts its cardiotonic action through a low affinity interaction with membrane phospholipids. Heterogeneous phospholipid vesicles also bound taurine with low affinity and positive cooperativity. The affinity of taurine for these mixed vesicles was enhanced by the inclusion of cholesterol. Taurine associated in a maximum ratio of 1:1 with homogeneous vesicles of phosphatidylcholine or phosphatidylserine. Vesicles of phosphatidylethanolamine bound taurine in a maximum ratio of 2:1, whereas those of phosphatidylinositol bound insignificant amounts of taurine.
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