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Regulation of Ca^{2+} fluxes across the sarcolemma and sarcoplasmic reticulum (SR) are critical for regulation of rhythm, contraction and relaxation in a beating heart. The $InsP_4$ stimulated Ca^{2+} transporter has been proposed to aid in the regulation of calcium across the myocardial membranes. Studies were performed on the $InsP_4$ stimulated Ca^{2+} transporter from both canine and bovine heart tissue to determine the location and ionic conditions under which the $InsP_4$ stimulated Ca^{2+} transporter works most efficiently. The results indicated that the $InsP_4$ stimulated Ca^{2+} transporter is present in the sarcolemma, and is functional under conditions that are physiologically relevant. These findings may compliment future *in vivo* or *in situ* studies that will further examine the role of the $InsP_4$ stimulated Ca^{2+} transporter in the heart.

THE INOSITOL TETRAKISPHOSPHATE STIMULATED CALCIUM TRANSPORTER IN CARDIAC MEMBRANES

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CHAPTER I

INTRODUCTION

One of the most critical processes in the heart is the regulation of Ca^{2+} across the sarcolemmal membrane. Although several of the mechanisms for fine control of Ca^{2+} influx or efflux have been carefully delineated, it is suggested here that there is an inositol tetrakisphosphate ($InsP_4$) stimulated Ca^{2+} transporter with properties of a Ca^{2+} carrier, which aids in the influx of Ca^{2+} across the cardiac sarcolemma (SL). Understanding the underlying mechanisms of the $InsP_4$ stimulated Ca^{2+} transporter and its potential role in regulating Ca^{2+} fluxes of the heart may be crucial for identifying future target areas for regulation of cardiac function.

The entry of external calcium into a heart cell is essential for several processes. During excitation and contraction of a heart cell, entry of Ca^{2+} into the cell via the L-type or T-type Ca^{2+} channels and, to a lesser extent, the Na/Ca exchanger (Litwin et al., 1998; Sheu et al., 1986) leads to the release of Ca^{2+} from the sarcoplasmic reticulum (SR) in a process termed calcium-induced calcium-release. Although the majority of the intracellular calcium initiating a contraction comes from the calcium-induced SR calcium release, a sustained inward calcium flow that persists throughout much of the contraction and lengthens the refractory period has been identified (Lee et al., 1984). The lengthening

of the refractory period by inward calcium currents is essential for the ability of the myocardium not to prematurely, and inefficiently, contract (Lee et al., 1984). Lee et al. (1984) and Hume and Giles (1983) identified this slow inward Ca^{2+} current ($i_{si,3}$)during the cardiac action potential that is distinct from the fast calcium current and calcium induced inward calcium current. This inward calcium flux does not appear to be blocked by any of the traditional calcium channel blockers, such as cadmium, and remains throughout most of the action potential. The amplitude of the $i_{si,3}$ is very small, but is capable of generating a long lasting calcium dependent action potential in the absence of any other inward current (Lee et al., 1984). It may be possible that a Ca^{2+} carrier regulated by $InsP_4$ and ATP may account for this slow inward Ca^{2+} current.

$InsP_4$ has been implicated in the regulation of cellular Ca^{2+} in several tissues by diverse mechanisms. Irvine and Moor (1986, 1987) suggested $InsP_4$ acts to control the size of the $InsP_3$ sensitive Ca^{2+} pool via regulation of Ca^{2+} influx across the sarcolemma. Morris et al. (1987) and Changya et al. (1989) also suggested a role for $InsP_4$ in regulating Ca^{2+} fluxes between intracellular compartments. Evidence for direct mobilization of Ca^{2+} by $InsP_4$ also exists. In microsomes from rat cerebellum, $InsP_4$ released calcium, although it was approximately thirty times less potent that $InsP_3$ (Joseph, 1989). Irvine et al. (1988) also reported that $InsP_4$ is additive with $InsP_3$ in inducing Ca^{2+} release from pituitary membrane preps. Hill et al. (1988) and Hill and Boynton (1990) found that $InsP_4$ induces sequestration of Ca^{2+} into $InsP_3$ sensitive pools in rat liver epithelial cells.

$InsP_4$ may influence potassium currents as well. Molleman (1991) postulates that inositol phosphates, namely inositol trisphosphate ($InsP_3$) and inositol tetrakisphosphate ($InsP_4$), may play a role in activation of calcium dependent potassium ion channels in the plasma membrane of DDT_1 MF-2 smooth muscle cells. In addition, $InsP_4$ acts to sustain a Ca^{2+} dependent K^+ current (Changya et al., 1989; Morris et al., 1987) in mouse lacrimal acinar cells. In HL-60 macrophage cells, there is a K^+ current induced directly by the addition of $InsP_4$ (Wu et al., 1991).

In *Xenopus laevis* oocytes, intracellularly administered $InsP_4$ sustains a slow inward Cl^- current (Mahlmann, 1989) elicited by thyrotropin releasing hormone (TRH). Parker and Miledi (1987) also observed that $InsP_4$ stimulated Cl^- currents in *Xenopus* oocytes. This chloride current was dependent upon intracellular Ca^{2+}. Interestingly, Parker and Miledi (1987) noted that $InsP_4$ was able to stimulate Ca^{2+} influx across the sarcolemma, as well as being able to mobilize intracellular calcium. Ferguson et al. (1991) noted that injection of $InsP_4$ into the oocyte released Ca^{2+} immediately, but lagged several minutes before simulating a Cl^- current. The Cl^- current produced, Ferguson notes, are oscillations and, due to the lag time between the rise in intracellular Ca^{2+} and the Cl^- oscillations, are not dependent upon Ca^{2+}.

In the heart, the role of inositol phosphates are not well established. However, a multitude of receptor systems in the heart have been shown to be coupled to inositol

phosphate pathways. For instance, increases in $InsP_3$ levels have been shown following alpha-adrenergic (Scholz et al., 1992; Poggioli et al., 1986) and muscarinic (Berg et al., 1989) receptor stimulation. Other receptor systems coupling to $InsP_3$ pathways in the heart include endothelin receptors (Sakurai et al., 1992; Suzuki et al., 1993), angiotensin II receptors (Sechi et al., 1992), serotonin receptors (Hamamori et al., 1990) and alpha thrombin receptors (Chein et al., 1990; Steinberg et al., 1991). In addition, several growth factors, including platelet derived growth factor, epidermal growth factor, fibroblast growth factor, insulin-like growth factor and transforming growth factor (Long et al., 1991; Fuller et al., 1992), have been implicated in PLC-gamma activation and subsequent stimulation of the phosphatidylinositol pathway in the heart.

$InsP_4$ has been identified in the heart (Kohl et al., 1990). In addition, the breakdown product of $InsP_4$, 1,3,4 $InsP_3$, (Guse et al., 1989; Renard and Poggioli, 1987; Kohl et al., 1990) and the $InsP_3$-kinase required for $InsP_4$ formation are present (Woodcock et al., 1993; Vanweyenberg et al., 1995). Huisaman et al.(1996) characterized $InsP_4$ binding to myocardial membranes. Three distinct binding sites, a low affinity binding site on the sarcoplasmic reticular membranes, and both a low affinity and high affinity binding site in sarcolemmal membranes, were found (Huisamen et al., 1996). These sites bind $InsP_4$ preferentially and do not represent $InsP_4$ binding to $InsP_3$ receptors (Huisamen et al., 1996). The presence of specific $InsP_4$ binding sites in myocardial membranes suggest a physiological role for $InsP_4$. $InsP_4$ has also been reported to stimulate Ca^{2+} transport in membranes with properties of cardiac junctional

SR vesicles (Quist et al., 1994a). However, definitive studies have not been completed to confirm the location of this calcium transporter. Several studies have suggested that $InsP_4$ is important in maintaining the positive inotropic effect of alpha-1 adrenergic stimulation (Kohl et al., 1990; Scholz et al., 1992; Guse et al., 1989). Although the evidence is somewhat indirect, these researchers have suggested that $InsP_4$ may be responsible for maintaining the positive inotropic effect as determined from comparison of the time courses of the formation of $InsP_4$ with the contractile response (Kohl et al., 1990; Scholz et al., 1992; Guse et al., 1989). Time course studies using muscarinic cholinergic agonists, which also stimulate the inositol phosphate pathway, have shown increasing levels of $InsP_4$ following agonist stimulation (Berg et al., 1989). Dassouli et al. (1993) measured stretch-induced cardiomyocyte production of $InsP_3$ and $InsP_4$. They found that increased production of $InsP_4$ may lead to a length dependent activation of cardiac muscle. Inositol polyphosphates may also be involved in increasing the Ca^{2+} sensitivity of the contractile proteins of the myocyte (Endo et al., 1988). Because of the potential role for $InsP_4$ in calcium regulation in other tissues and in the heart, there is reason to continue to study the effects of $InsP_4$ on the heart where calcium regulation is crucial for function.

CHAPTER II

MATERIALS AND METHODS

Preparation of microsomes and purified membrane vesicles from bovine ventricular tissue. Bovine ventricular tissue was obtained from Hamilton's Meat Market in Weatherford, TX. Microsomes were prepared using a modification of the method used by Quist et al.(1994b). To prepare the microsomes, approximately 300g of ventricular tissue was minced and washed in ice cold isotonic saline (150 mM NaCl, 0.1 mM EGTA). Tissue was suspended in 1 L of homogenizing buffer (10 mM HEPES, pH 7.4, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.5 mM dithiothreitol) using a large Kinematica Polytron homogenizer for 6 passes, 10 seconds per pass, at a setting of 6. In one preparation, large particles were evident, so the homogenate was subjected to an additional 2 passes for 10 seconds at a setting of 5 with a Brinkmann Polytron homogenizer. In the second preparation, this additional step was unnecessary because no large particles were evident. Homogenates were then poured into 250 mL centrifuge tubes and centrifuged at 7000 r.p.m. for 15 minutes at 5° C. The supernatant was then poured into 50 mL centrifuge tubes and centrifuged at 17 K for 20 minutes at 5° C to sediment microsomal membranes. The supernatant from the 17 K, 20 minute centrifugation was discarded and the remaining pellet was resuspended with 1 mL of homogenizing buffer and transferred to a 100 mL beaker. The tubes were washed with 0.5 mL of homogenizing buffer and that wash was

transferred to the beaker as well. The pellets were resuspended with a Potter-Elvehjem homogenizer, 5 passes at a setting of 5. The microsomes were divided into aliquots and stored at -80° C. Protein was determined by using the Bradford method (Bradford, 1976).

Sucrose gradient membranes were prepared by loading 7 mg of thawed bovine microsomes onto a gradient containing 8 mL of 40% sucrose, 7 mL of 30% sucrose and 7 mL of 22.5% sucrose. This gradient was then centrifuged at 17 K for 1 hour at 5° C in a swinging bucket rotor. The membrane bands on top of the sucrose layers were transferred into 50 mL centrifuge tubes and had 20-25 mL of HB added. They were centrifuged at 17 K for 20 minutes at 5° C. The resulting pellets had 4.5 mL of HB added and were resuspended using a Potter-Elvehjem, 12 passes at a setting of 3. Aliquots were frozen at -80° C until use. Protein was determined using the Bradford method (Bradford, 1976).

To prepare sarcolemmal vesicles a modified method by Quist et al. (1989) was used. Briefly, 48 g of tissue was divided into twelve 50 mL tubes (4 g/ tube), minced in isotonic saline with scissors, 0.1 mM EGTA centrifuged at 1000 r.p.m. for 5 minutes at 5° C and had the supernatant aspirated off. The tissue was resuspended with 20 mL of homogenizing buffer, centrifuged as above and supernatant was aspirated off. The tissue was resuspended in 18 mL of homogenizing buffer and homogenized with a Polytron homogenizer (4 passes for 6 seconds at a setting of 5). The homogenate was pipetted on a gradient made up of 6 mL each of 22.5% sucrose and 8% sucrose. Tubes were

centrifuged at 17 K for 1 hour at 5°C. The membrane layer between the sucrose layers was collected and transferred to 50 mL centrifuge tubes. Then, 20-25 mL of homogenizing buffer was added and centrifuged at 19 K for 30 minutes at 5° C. The supernatant was aspirated off, and the pellets were each resuspended with 3 mL of homogenizing buffer, combined and suspended with 3-4 passes by Potter-Elvehjem. Protein was determined by using the Bradford method (Bradford, 1976).

Preparation of microsomes and purified membranes vesicles from canine ventricular tissue. Canine microsomes were prepared according to Quist et al.(1994b). Briefly, 4 g of tissue was put in each 50 mL centrifuge tube. The tissue was finely minced with scissors in the presence of isotonic saline and 0.1 mM EGTA and washed with the same solution to remove any blood. The tissue was then homogenized in 18 mL of homogenizing buffer (HB) with a Polytron, 4 passes for 10 seconds each at a setting of 5. Following this, 13 mL of HB was added and then centrifuged at 8 K for 15 minutes at 5° C. The supernatant was collected into 50 mL tubes and centrifuged at 17 K for 20 minutes at 5° C. The supernatant was discarded and the remaining pellet was loosened with 2 mL of HB, combined into 1 tube and resuspended using a Polytron, 2 passes for 10 seconds at a setting of 5. Aliquots were stored stored at -80° C until use. Protein was determined using the Bradford method (Bradford, 1976).

Sucrose gradient membranes were prepared by loading ~6 mg of canine microsomes onto a gradient containing 8 mL of 40% sucrose, 7 mL of 30% sucrose and 7

mL of 22.5% sucrose. This gradient was then centrifuged at 17 K for 1 hour at 5° C in a swinging bucket rotor. The membrane bands on top of the sucrose layers were transferred into 50 mL centrifuge tubes and 20-25 mL of HB was added. The tubes were centrifuged at 17 K for 20 minutes at 5° C. The supernatant was aspirated off and resulting pellets were resuspended with 4.5 mL of HB using a Potter-Elvehjem homogenizer (12 passes at a setting of 3). Aliquots were frozen at -80° C. Protein was determined using the Bradford method (Bradford, 1976).

Calcium uptake. After thawing the samples at room temperature, aliquots of membranes (approximately 25-50 μg/tube) were suspended in isotonic medium containing 100 mM KCl, 30 mM choline chloride, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM NaN_3, 20 mM HEPES pH 7.4 and 2.5 mM potassium oxalate. The final volume varied according to how many tubes were used, but on average, the final volume was 150 μL. This was considered to be phase I. The membrane containing medium was then kept for 10 minutes on ice. Aliquots (between 0.5 and 1.5 μg of protein/tube) were then suspended in $^{45}Ca^{2+}$ uptake medium (15μL from phase 1 into 60 μL) containing control buffer (similar to that above except with 148 nM free $^{45}Ca^{2+}$ and 10 mM oxalate) or control buffer supplemented with 10 μM 3,4,5,6, and incubated for variable times at 30° C. The final volume of phase II was 75 μL. Ca^{2+} uptake was stopped with 25 μL of a stop solution containing solution 0.8 mM La^{3+} and 150 mM choline chloride. Aliquots were filtered on 0.45 μM Millipore filters and washed 3 x 3 mL with wash solution containing 150 mM choline chloride, 10 mM HEPES, pH 7.4, and 0.1 mM $LaCl_3$ as

described (Quist et al., 1994a). The filters were placed in EcoLume and counted in a Packard Scintillation Counter. In some experiments, ionic conditions or nucleotide concentrations were changed to observe changes in Ca^{2+} uptake ability.

In some calcium uptake experiments, the reaction was carried out in the first phase (phase I) by including the $^{45}Ca^{2+}$, $InsP_4$, ATP or various other agents in the phase I incubation media since there would be no additional dilution into a calcium uptake medium as described above (phase II). The tubes containing the incubation media, membranes (4-6 μg of protein/tube) and test agents (final volume of 50 μL) were incubated for 5 minutes at 30° C, stopped with 0.8 mM $LaCl_3$ and 150 mM choline chloride containing medium, filtered and counted as above. In some experiments, incubation time, Ca^{2+} concentration or other ionic parameters were changed.

Ion requirement studies. Ion requirement studies were performed using bovine or canine vesicles. Calcium uptake (as described above) was assayed by substituting various intra- and extra-cellular concentrations of potassium (0-150 mM), sodium (0-150 mM), choline (0-150 mM), oxalate (0-12 mM), chloride (0-150 mM), hydrogen (pH 6-8) and magnesium (0-10 mM). These conditions were modified through the use of different ion solutions.

Nucleotide selectivity. Various nucleotides, including ATP, ADP, GDP and AMP were tested for their ability to affect the Ca^{2+} transporter under established Ca^{2+} uptake or

efflux conditions. The range of concentrations studied were between 0-3mM for the nucleotides.

Na-K-ATPase activity. Samples of membranes were added to a mixture containing 25 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 0.1 mM EGTA, 37.5 μg of saponin and ± 1 mM Ouabain. The above mixture was incubated for 30 minutes at 37° C. Following incubation, 1 mL of 2% SDS was added to each tube. Then, 1 mL of ammonium molybdate solution and 0.2 mL of ANS reagent were added to the tubes and vortexed. The color was read 15-30 minutes later in a Hitachi spectrophotometer at a wavelength of 700 nm.

Materials. Inositol tetrakisphosphate (InsP4) 3,4,5,6 was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. EcoLume scintillation fluid was purchased from ICN, Costa Mesa, CA, U.S.A. All other chemical used were of reagent grade.

Statistical analysis. Data contained is presented ± the standard error of the mean of at least three replicate measurements on the same tissue. Key results were replicated in separate experiments using different tissue sources.

CHAPTER III

RESULTS

Subcellular Localization of $InsP_4$ stimulated Ca^{2+} transport- The subcellular localization of the $InsP_4$ stimulated Ca^{2+} transporter was accomplished by using several membrane fractionation techniques. Canine and bovine vesicles were assayed for $InsP_4$ or ATP stimulated Ca^{2+} uptake in the presence of 10 mM potassium oxalate and 4.62 μM Ca^{2+} (Fig. 1 and 2). In bovine SL membranes, $InsP_4$ stimulated Ca^{2+} uptake was 2.5 times greater than in microsomal membranes. In addition, the background, as well as the ATP stimulated Ca^{2+} uptake, was greater in bovine SL membranes than microsomal membranes.

In bovine vesicles, $InsP_4$ stimulated Ca^{2+} uptake was highest in the SL fraction. In canine membrane vesicles separated on a sucrose density gradient, the fraction enriched in sarcolemma (22.5% sucrose) had the highest $InsP_4$ stimulated Ca^{2+} uptake. In contrast, the canine 22.5% sucrose membranes were lowest in ATP dependent Ca^{2+} uptake. Canine vesicles derived from the 30 and 40% sucrose gradient were approximately three fold higher in ATP (0.5 mM) dependent Ca^{2+} uptake versus $InsP_4$ stimulated Ca^{2+} uptake. The higher level of ATP dependent Ca^{2+} uptake indicates that the canine 30 and 40%

sucrose gradient membranes were derived from the sarcoplasmic reticulum (SR), which is rich in sarcoplasmic reticulum Ca-ATPase (SERCA).

$InsP_4$ stimulated versus ATP dependent Ca^{2+} uptake- $InsP_4$ and ATP stimulated Ca^{2+} uptake were examined in canine 22.5% sucrose gradient membranes (Fig. 3). The data shown in figure 3 were obtained using a phase I experimental protocol (see methods). Basal levels of calcium uptake were determined by incubating membranes with $^{45}Ca^{2+}$ in the absence of any Ca^{2+} uptake stimulators. Thapsigargin was added in another group to show that the basal levels of calcium uptake were not suppressed by thapsigargin, a SERCA inhibitor. The effect of 1 mM ATP on Ca^{2+} uptake was to produce a 2.7 fold increase over basal Ca^{2+} uptake. The addition of 5 μM thapsigargin completely inhibited ATP dependent uptake. $InsP_4$ (10 μM) induced a 2 fold increase in Ca^{2+} uptake over basal uptake that was not suppressed by thapsigargin. When 10 μM $InsP_4$ and 1 mM ATP were added together, the level of Ca^{2+} uptake was equivalent to ATP stimulated Ca^{2+} uptake alone. Therefore, the effects of ATP and $InsP_4$ were not additive. Later results indicate that $InsP_4$ stimulated Ca^{2+} uptake is abolished in the presence of 1 mM ATP. Addition of 5 μM thapsigargin to the ATP/$InsP_4$ mixture completely inhibited Ca^{2+} uptake. These results suggest that $InsP_4$ and ATP may stimulate calcium uptake by different mechanisms; ATP via stimulation of SERCA and $InsP_4$ by the proposed $InsP_4$ stimulated Ca^{2+} transporter. However, ATP (1 mM) may block $InsP_4$ stimulated Ca^{2+} uptake, possibly into a different membrane compartment.

Na-K-ATPase activity- To identify the membranes studied, Na-K-ATPase activity ± 1 mM ouabain was assayed in bovine 22.5, 30 and 40% sucrose gradient membranes (Fig. 4). Na-K-ATPase is a sarcolemmal marker. The highest activity was located in the 22.5% sucrose gradient membrane fraction. This links the 22.5% sucrose gradient membranes with the sarcolemmal membranes of the cell. This pattern of activity agrees with other Na-K-ATPase data from our lab on 22.5, 30, 40% gradient membranes from the dog and rat (data not shown). Because the 22.5% membranes are associated with the highest Na-K-ATPase activity and also with the highest $InsP_4$ stimulated Ca^{2+} uptake, it is concluded that the $InsP_4$ stimulated Ca^{2+} transporter is located in sarcolemmal membranes.

Fig. 1. $InsP_4$ stimulated Ca^{2+} uptake and ATP dependent Ca^{2+} uptake in bovine microsomes and SL membranes. Bovine microsomal and SL membranes were assayed for Ca^{2+} uptake ability in the presence or absence of 10 μM $InsP_4$ or 0.1 mM ATP for 10 minutes at 30° C according to Materials and Methods.

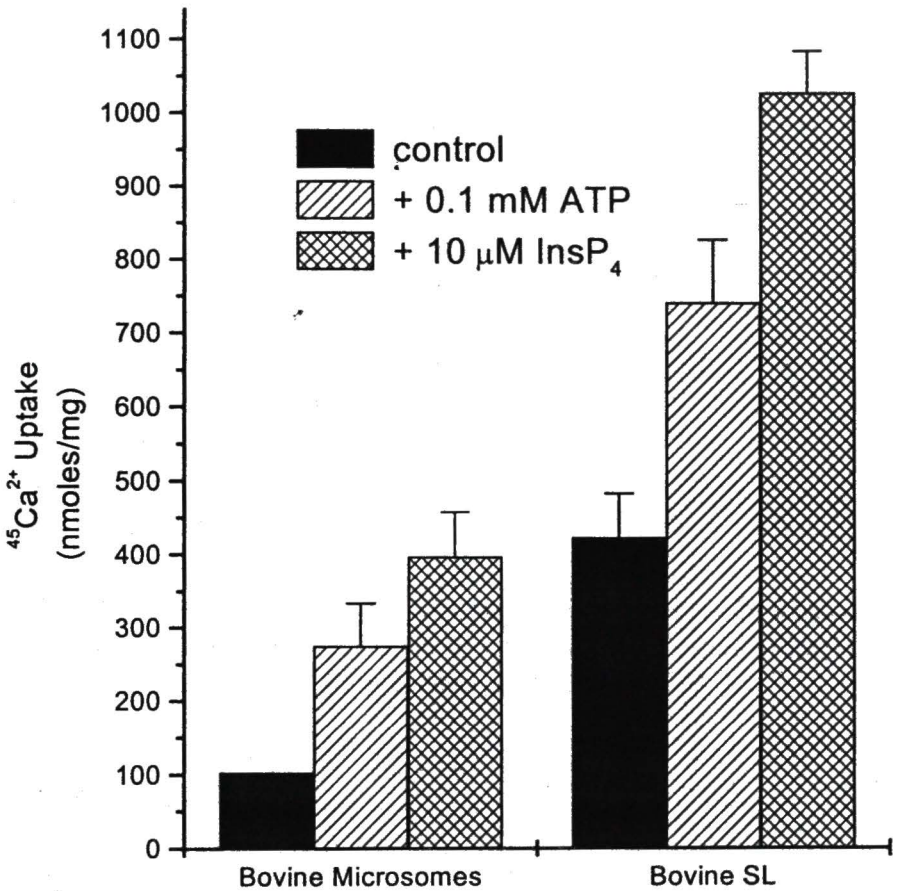
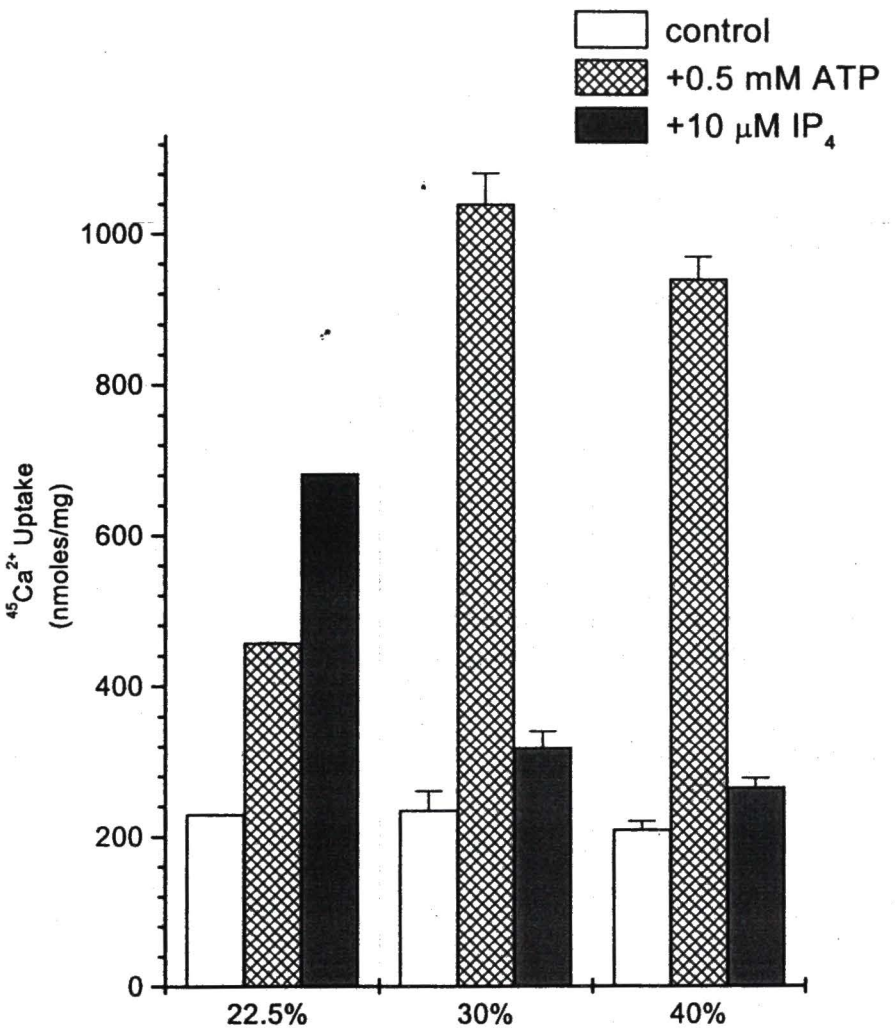

1100
1000
900
800
700
600
500
400
300
200
100
0
$^{45}Ca^{2+}$ Uptake
(nmoles/mg)
control
+ 0.1 mM ATP
+ 10 μM $InsP_4$
Bovine Microsomes
Bovine SL

Fig. 2. $InsP_4$ stimulated and ATP dependent Ca^{2+} uptake in canine sucrose gradient membranes. Canine sucrose gradient membranes were assayed for 10 minutes at 30° C in the presence or absence of 10 μM $InsP_4$ or 0.5 mM ATP.



Canine sucrose gradient membranes

Fig. 3. Thapsigargin (TG) sensitivity on $InsP_4$ stimulated and ATP dependent Ca^{2+} uptake in canine 22.5% sucrose gradient membranes. Canine 22.5% membranes were assayed using a phase I protocol (see Materials and Methods) for 5 minutes at 30° C.

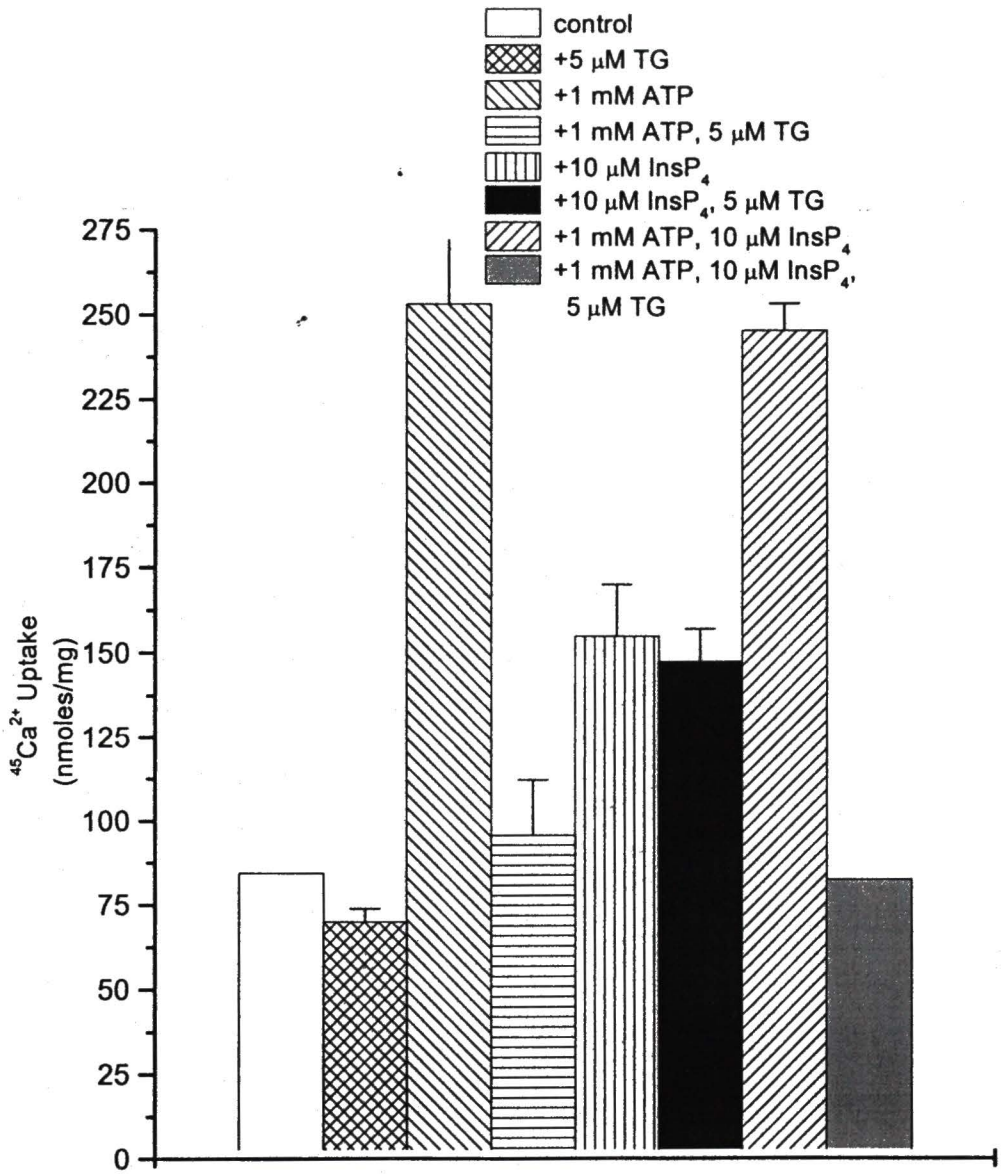
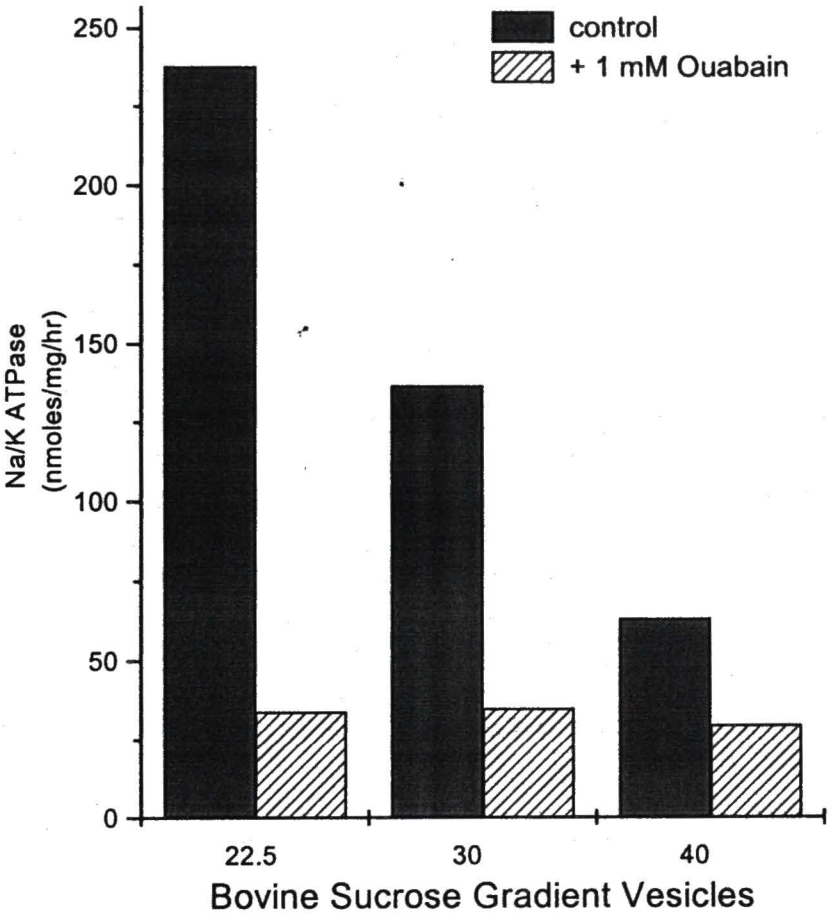

control
+5 μM TG
+1 mM ATP
+1 mM ATP, 5 μM TG
+10 μM InsP4
+10 μM InsP4, 5 μM TG
+1 mM ATP, 10 μM InsP4
+1 mM ATP, 10 μM InsP4, 5 μM TG
45Ca2+ Uptake (nmoles/mg)
275
250
225
200
175
150
125
100
75
50
25
0

Fig. 4. Na/K ATPase activity in Bovine 22.5, 30 and 40% sucrose gradient membranes. Na/K ATPase activity was measured according to Materals and Methods.


control
+ 1 mM Ouabain
Na/K ATPase
(nmoles/mg/hr)
250
200
150
100
50
0
22.5
30
40
Bovine Sucrose Gradient Vesicles

ATP effects on Ca^{2+} uptake- Figure 5 depicts the ATP concentration curves in bovine microsomal and sarcolemmal (SL) vesicles. There is a large uptake of calcium at lower concentrations of ATP (<0.33 mM) for bovine microsomes and SL. At greater ATP concentrations, Ca^{2+} uptake is progressively decreased. ATP data obtained from the 22.5% gradient canine vesicles (Fig. 6a) indicate that ATP levels 0.33 mM or lower will stimulate Ca^{2+} uptake. ATP concentrations above 0.5 mM causes a decrease in Ca^{2+} uptake. This decrease in Ca^{2+} uptake seems to be more pronounced in bovine vesicles. The preparations more enriched in ATP dependent Ca^{2+} uptake activity and SERCA have more of a blunted response to the high levels of ATP presumably because the SERCA can pump against a concentration gradient and load into SR vesicles.

Another interesting phenomenon is that the ATP concentration curves for Ca^{2+} uptake depends upon what phase the ATP is added (Fig. 6a vs. Fig. 6b). Figure 6b represents a phase I ATP curve in which the experiment is performed without dilution into a separate Ca^{2+} uptake media (see methods). The decrease in Ca^{2+} uptake by high ATP is not as drastic in phase I conditions as compared to a phase II conditions (Figs. 6a and 6b). One possible explanation is that the higher membrane concentrations in phase I lend some resistance to the effects of high (>0.5 mM) ATP. I have replicated this result in the other canine sucrose fractions, canine microsomes, bovine microsomes and bovine SL vesicles.

Nucleotide selectivity on Ca^{2+} uptake ability- The effects of various nucleotides on Ca^{2+} uptake were examined. Figure 7 depicts the effects of different nucleotides on Ca^{2+} uptake. The effects of ATP, ADP, GDP and AMP on $InsP_4$ stimulated Ca^{2+} uptake in bovine SL vesicles were studied. In the absence of nucleotides, $InsP_4$ increased Ca^{2+} uptake 3.5 fold over basal Ca^{2+} uptake in the absence of $InsP_4$. The nucleotides were tested at concentrations of 0.33 mM and incubated for 2 minutes at 30° C in the presence of 10 mM magnesium. The 0.33 mM ATP containing group showed lower basal levels of Ca^{2+} uptake, as well as lower $InsP_4$ stimulated levels of Ca^{2+} uptake. The possible reasons for such an observation will be discussed in chapter 4. Neither GDP, nor AMP, altered the baseline or $InsP_4$ stimulated Ca^{2+} uptake significantly. ADP, however, stimulated Ca^{2+} uptake in the absence of $InsP_4$ (three fold) and also may have increased the effects of $InsP_4$ on Ca^{2+} uptake.

Effects of various ions on Ca^{2+} uptake - Figure 8 shows the effect if increasing potassium chloride concentration on basal and $InsP_4$ stimulated Ca^{2+} uptake in bovine microsomes. The ionic strength was maintained at 130 mM by substituting choline chloride. Potassium chloride concentrations ranged from 0-130 mM. Both the effects of 0.1 mM ATP and 10 μM $InsP_4$ are shown in addition to basal levels of calcium uptake. Although Ca^{2+} uptake increased with KCl concentration, both ATP dependent and $InsP_4$ stimulated Ca^{2+} uptake were present in the absence of potassium.

Figure 9 shows basal and $InsP_4$ stimulated Ca^{2+} uptake in the presence and absence of chloride ions in bovine microsomes. Potassium sulfate was substituted for potassium chloride. Although Ca^{2+} uptake was only half as large as the control $InsP_4$ value, K_2SO_4 containing tubes had a 2-3 fold increase in Ca^{2+} uptake with 10 μM $InsP_4$. When n-methyl-α-glucopyranoside was substituted for potassium, choline and chloride in a canine SL prep (Fig. 10), $InsP_4$ was still able to increase Ca^{2+} uptake by 40%. The basal levels of Ca^{2+} uptake in the n-methyl-α-glucopyranoside were 30% higher than in the ion containing group.

Sodium ions were also examined for their potential role in regulating the $InsP_4$ stimulated Ca^{2+} transporter. One of the possible locations of the $InsP_4$ stimulated Ca^{2+} transporter is in the transverse tubular portion of the sarcolemma. Because of the high density of Na-Ca exchange proteins in the sarcolemma (Durkin et al., 1991; Phillipson et al., 1988), sodium was thought to be a potentially important regulator. However, as figure 11 demonstrates, 66 mM NaCl did not enhance, but may have inhibited, $InsP_4$ stimulated Ca^{2+} uptake in canine 22.5% sucrose gradient vesicles. In the medium containing 130 mM KCL, there was a 101% increase in Ca^{2+} uptake with the addition of 10 μM $InsP_4$. In the medium containing 66 mM NaCl and 34 mM KCl, there was an 85% increase with $InsP_4$. There was no change between the Ca^{2+} uptake in the 1 mM ATP of the KCl control group and the 1 mM ATP of the 66 mM NaCl group. Although 0.1 mM ATP was not tested on the KCl control in this experiment, the 0.1 mM ATP stimulated Ca^{2+} uptake values of the NaCl containing tubes were not significantly different from a

similar experiment testing 0.1 mM ATP stimulated Ca^{2+} uptake in medium containing 130 mM KCl (results not shown).

The effect of pH on $InsP_4$ stimulated Ca^{2+} uptake was studied in canine SL vesicles. The pH range tested was between 6.5-8.0. These values were chosen as pH values that define the outer limits of physiological relevance. The level of $InsP_4$ stimulated Ca^{2+} uptake were: 19% at pH 6.5, 42% at pH 7.0, 60% at pH 7.4 and 61% at pH 8.0. The basal, as well as $InsP_4$ stimulated, Ca^{2+} uptake at pH 7.0 was significantly higher than at the other pH values. In another pH curve done on canine microsomes (data not shown), $InsP_4$ stimulated Ca^{2+} transport increased in a similar manner, with high background occurring at pH 7.0.

Fig. 5. The effect of ATP on Ca^{2+} uptake in bovine microsomal or SL membranes. ATP concentration curve (0-1.0 mM) was performed on bovine vesicles for 10 minutes at 30° C.

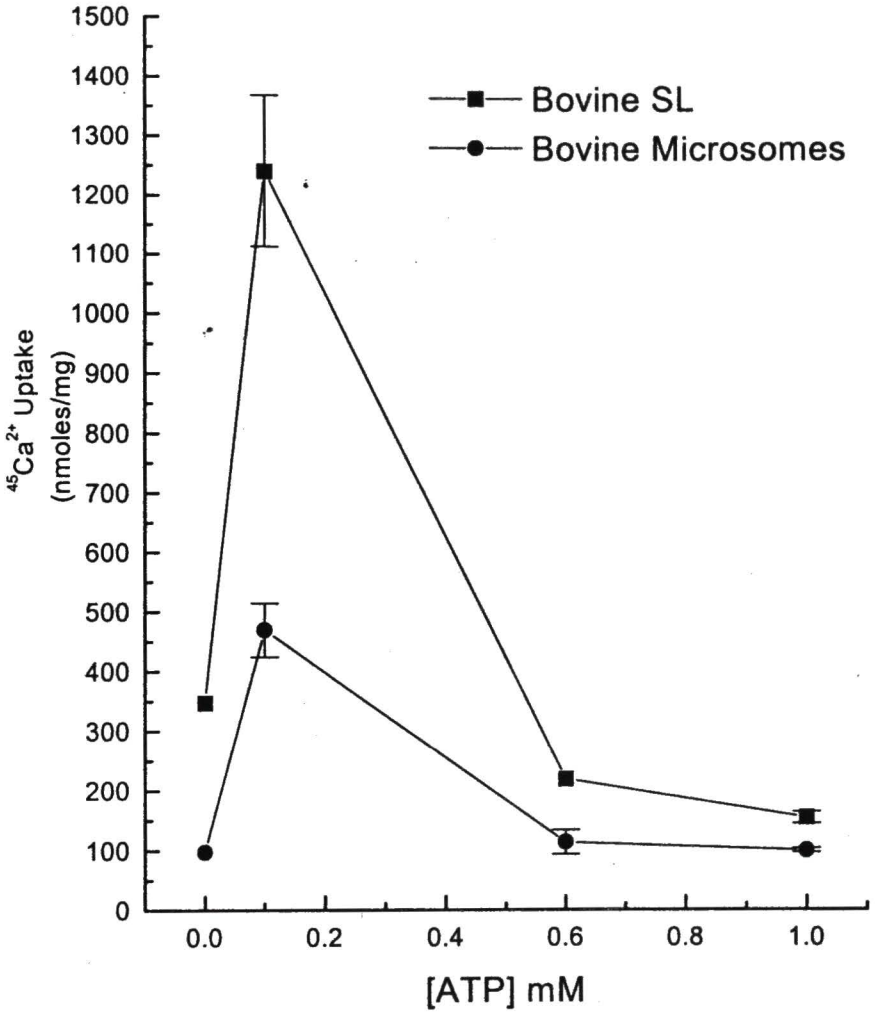

Bovine SL
Bovine Microsomes
$^{45}Ca^{2+}$ Uptake (nmoles/mg)
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
[ATP] mM

Fig. 6a. The effect of ATP on canine 22.5% sucrose gradient vesicles using a phase II experimental protocol. Canine 22.5% vesicles assayed Ca^{2+} uptake using a phase II protocol (standard) for 10 minutes at 30° C over a range of ATP concentrations (0-1.0 mM). See Materials and Methods.

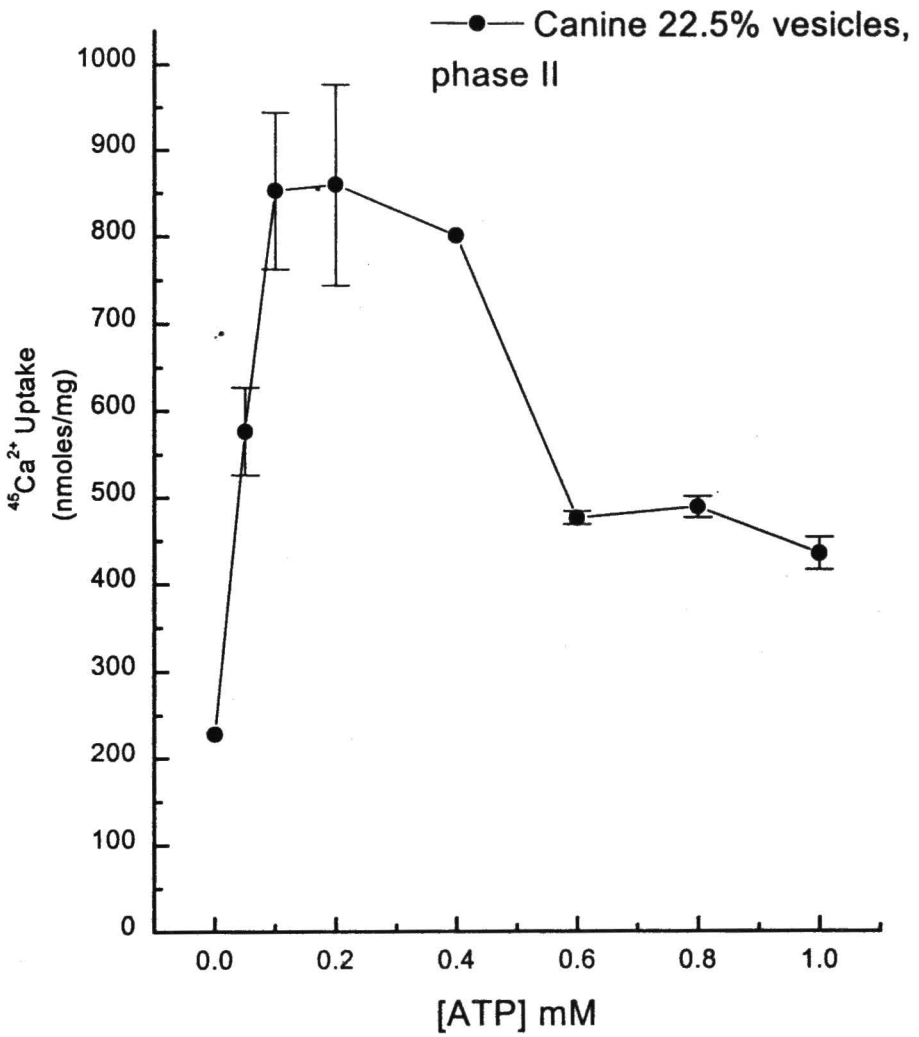

Canine 22.5% vesicles, phase II
$^{45}Ca^{2+}$ Uptake (nmoles/mg)
1000
900
800
700
600
500
400
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
[ATP] mM

Fig. 6b. Effects of ATP on canine 22.5% sucrose gradient membranes using a phase I experimental protocol. Canine 22.5% sucrose gradient membranes were assayed for Ca^{2+} uptake ability over 10 minutes at 30° C according to Materials and Methods.

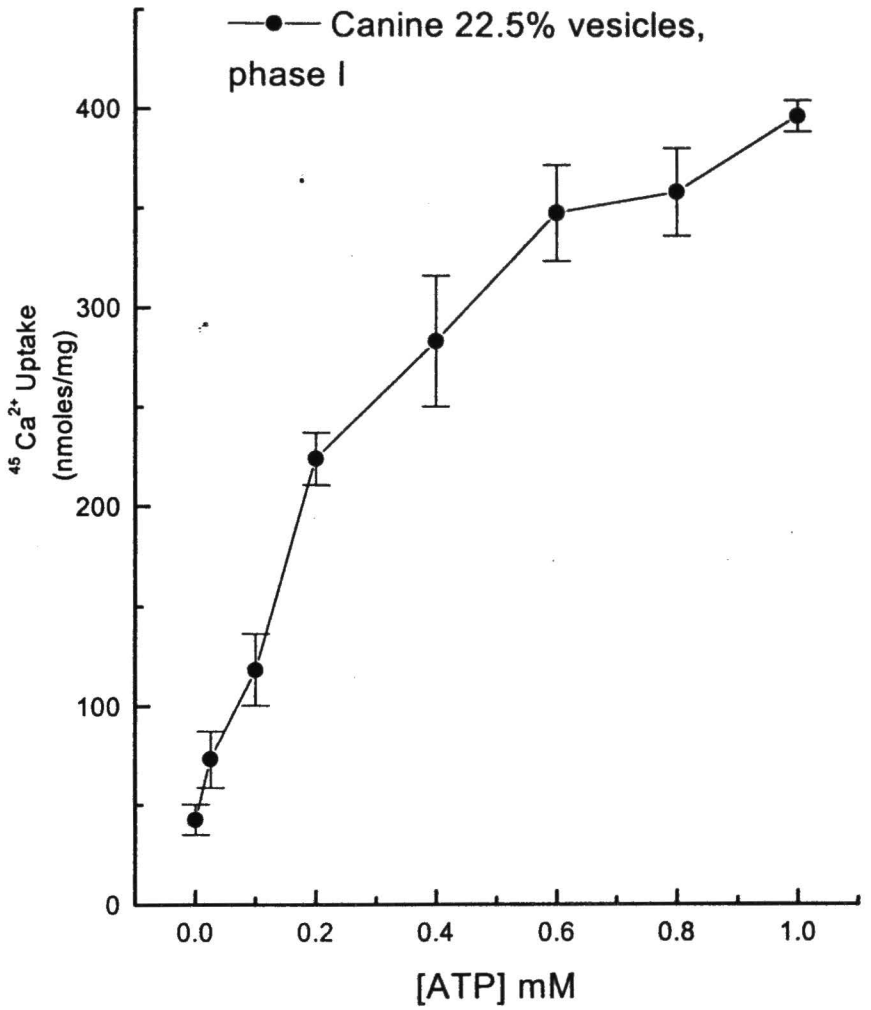

Canine 22.5% vesicles, phase I
400
300
200
100
0
$^{45}Ca^{2+}$ Uptake (nmoles/mg)
0.0
0.2
0.4
0.6
0.8
1.0
[ATP] mM

Fig. 7. Nucleotide selectivity and Ca^{2+} uptake in bovine SL membranes. Bovine SL membranes were incubated with 0.33 mM concentrations of various nucleotides and incubated for 2 minutes at 30° C in the presence or absence of 10 μM $InsP_4$.

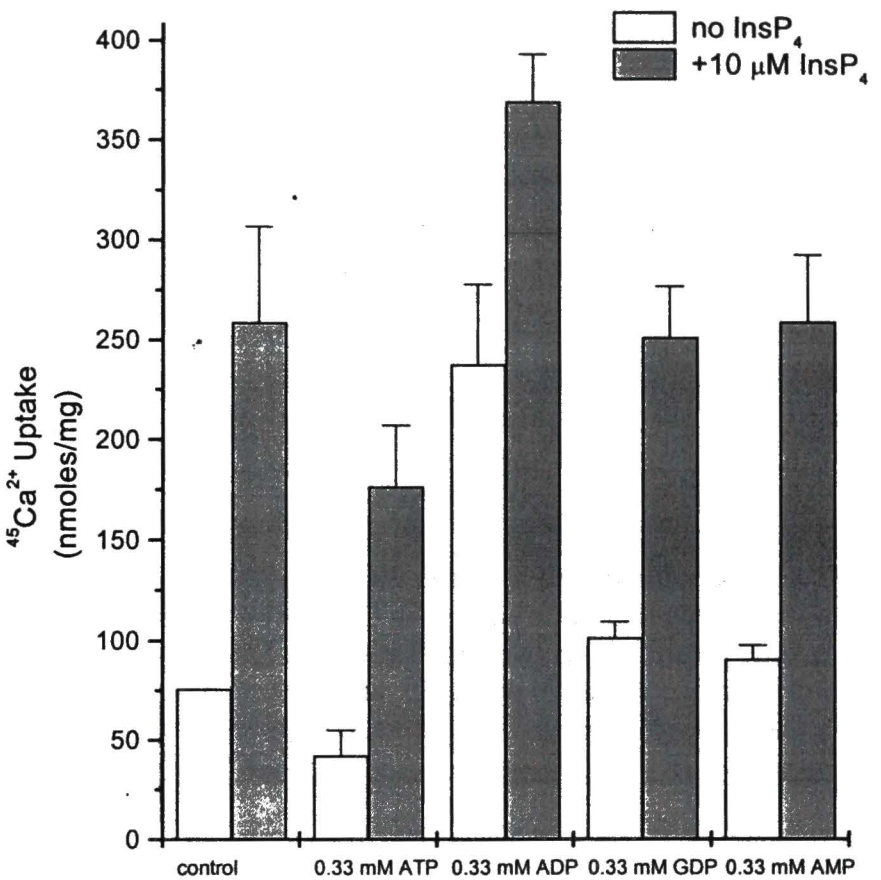

no InsP4
+10 μM InsP4
45Ca2+ Uptake
(nmoles/mg)
400
350
300
250
200
150
100
50
0
control
0.33 mM ATP
0.33 mM ADP
0.33 mM GDP
0.33 mM AMP

Fig. 8. Potassium chloride concentration curve in bovine microsomes. Potassium chloride concentration curve (0-130 mM) was performed on bovine microsomes in the presence and absence of 0.1 mM ATP. $InsP_4$ (10μM) results from a similar experiment using the same conditions are indicated on the graph by the independent filled squares. Choline chloride (0-130 mM) was used to balance ionic conditions. Uptake was performed for 2 minutes at 30° C.

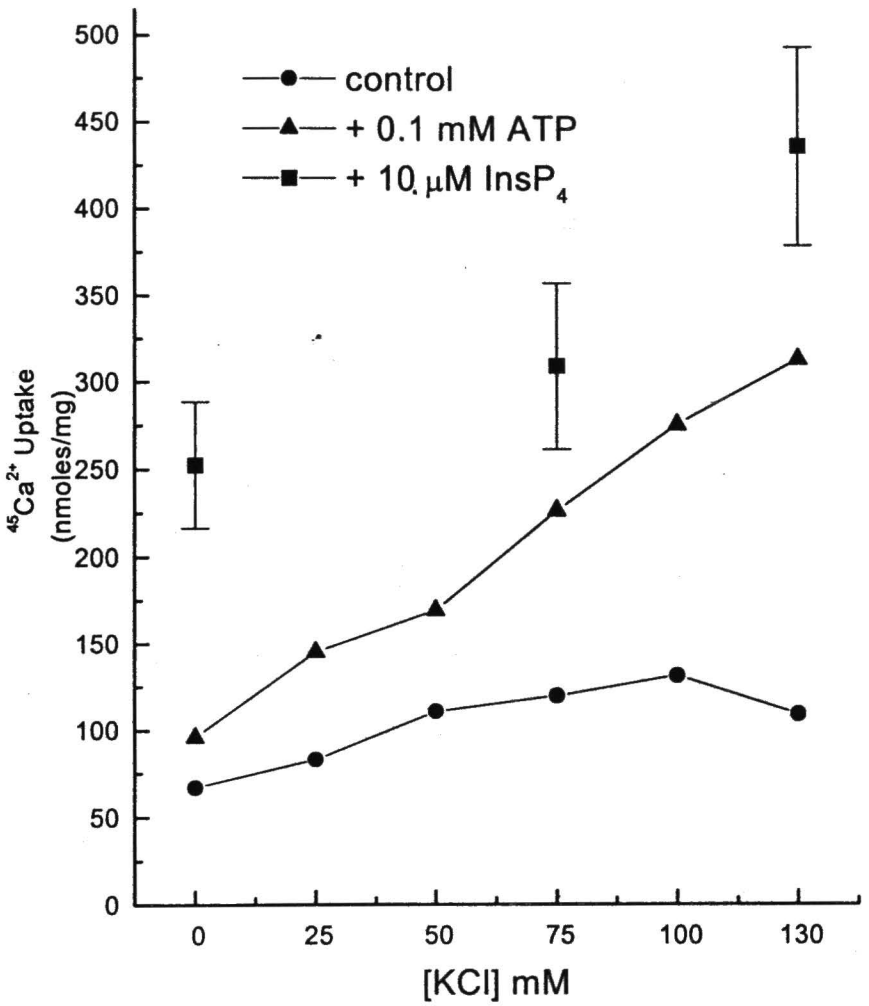

control
+ 0.1 mM ATP
+ 10 μM InsP4
45Ca2+ Uptake (nmoles/mg)
500
450
400
350
300
250
200
150
100
50
0
0
25
50
75
100
130
[KCl] mM

Fig. 9. Effects of chloride ion substitution on $InsP_4$ stimulated Ca^{2+} uptake in bovine microsomes. Potassium sulfate was substituted for chloride salts to test the effects of chloride ions on $InsP_4$ stimulated Ca^{2+} uptake in bovine microsomes. Uptake was performed for 2 minutes at 30° C. In the last group, 1 mM $MgCl_2$ was added back to the uptake media to observe any changes.

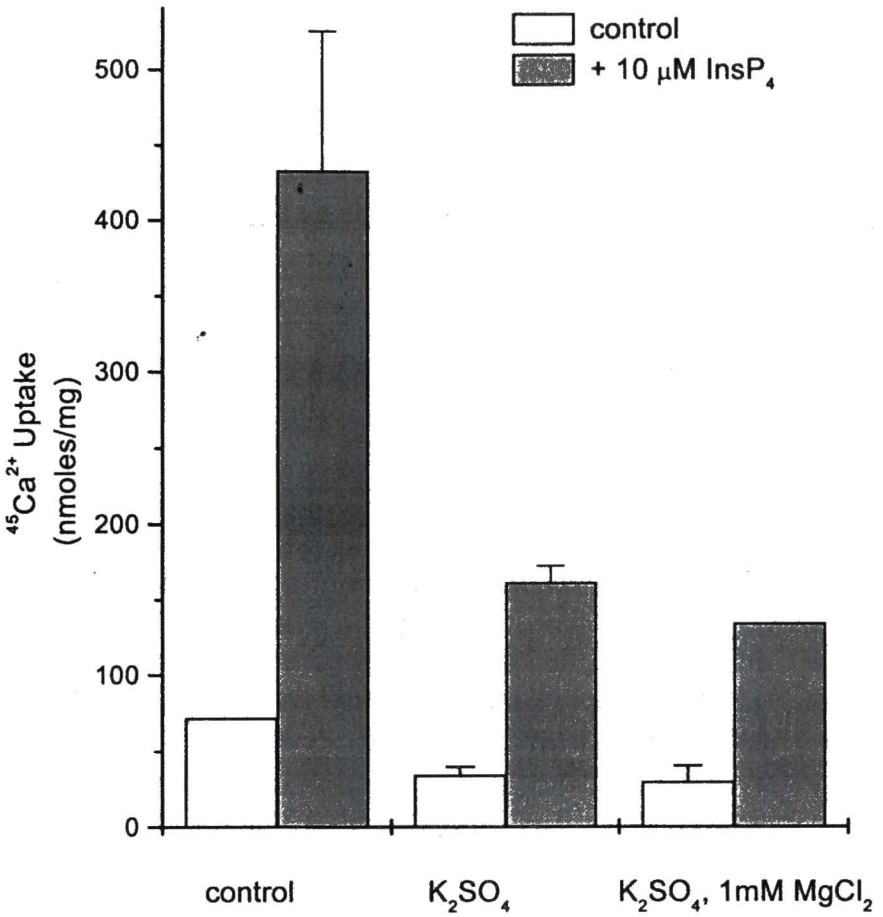

control
+ 10 μM InsP4
500
400
300
200
100
0
45Ca2+ Uptake
(nmoles/mg)
control
K2SO4
K2SO4, 1mM MgCl2

Fig. 10. Effect of ion substitution in canine SL membranes. All ions were substituted for by n -methyl- α -glucopyranoside in canine SL membranes and assayed for Ca^{2+} uptake ability in the presence and absence of 10 μM $InsP_4$. pH was 7.0 The control group contained HEPES buffer with ions added back in to the media (100 mM KCl, 20 mM Choline Chloride, 10 mM NaCl). There was a two minute incubation at 30° C.

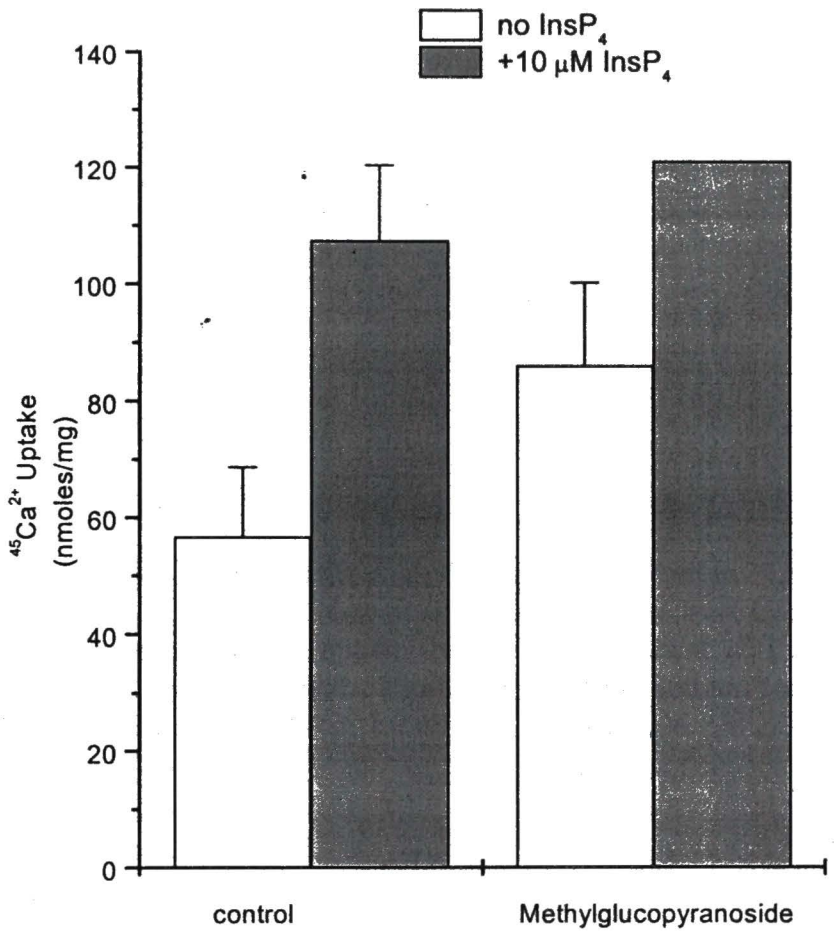

no InsP4
+10 μM InsP4
140
120
100
80
60
40
20
0
45Ca2+ Uptake
(nmoles/mg)
control
Methylglucopyranoside

Fig. 11. Sodium vs. Potassium medium on Ca^{2+} uptake in canine 22.5% sucrose gradient membranes. Canine 22.5% sucrose gradient vesicles were tested for $InsP_4$ stimulated and ATP dependent Ca^{2+} uptake ability for 5 minutes at 30° C. Potassium group tested a control, 1 mM ATP and a 10 μM $InsP_4$ group. Sodium contained 66 mM NaCl and tested control, 0.1 mM ATP, 1.0 mM ATP and 10 μM $InsP_4$ containing group.

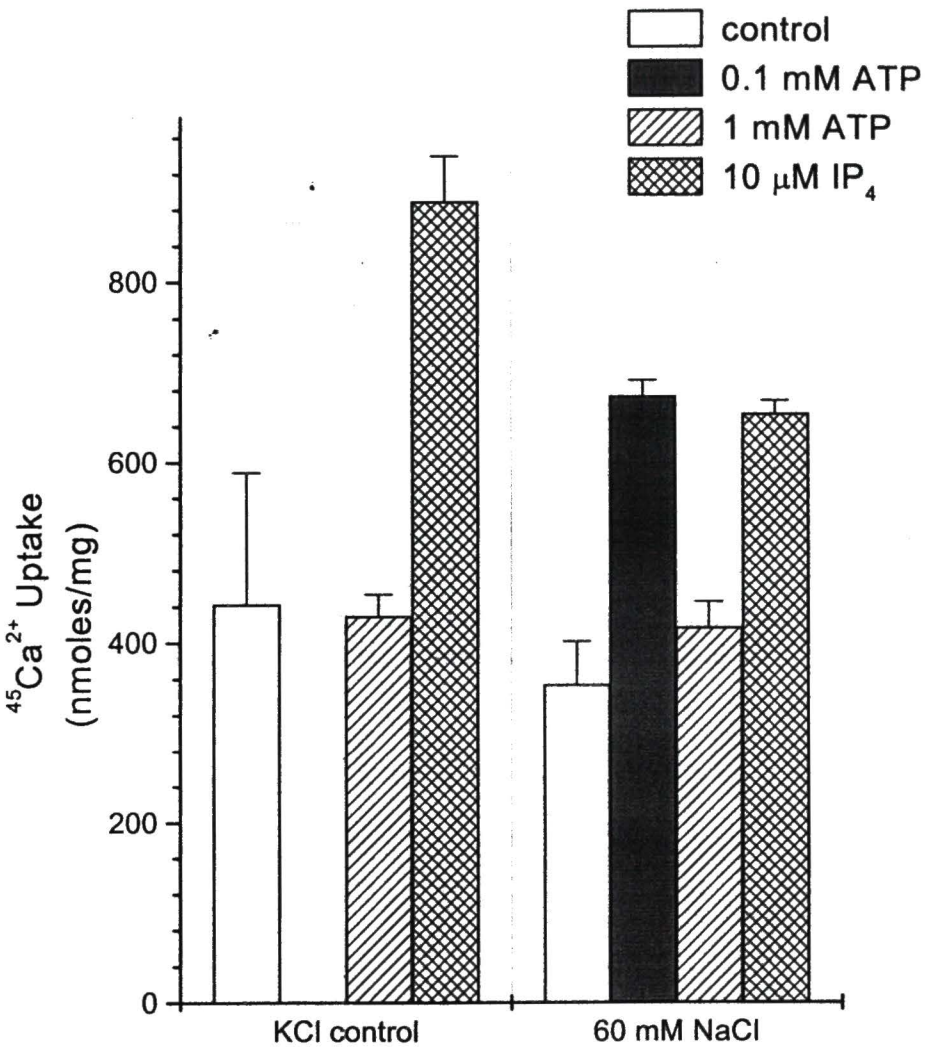
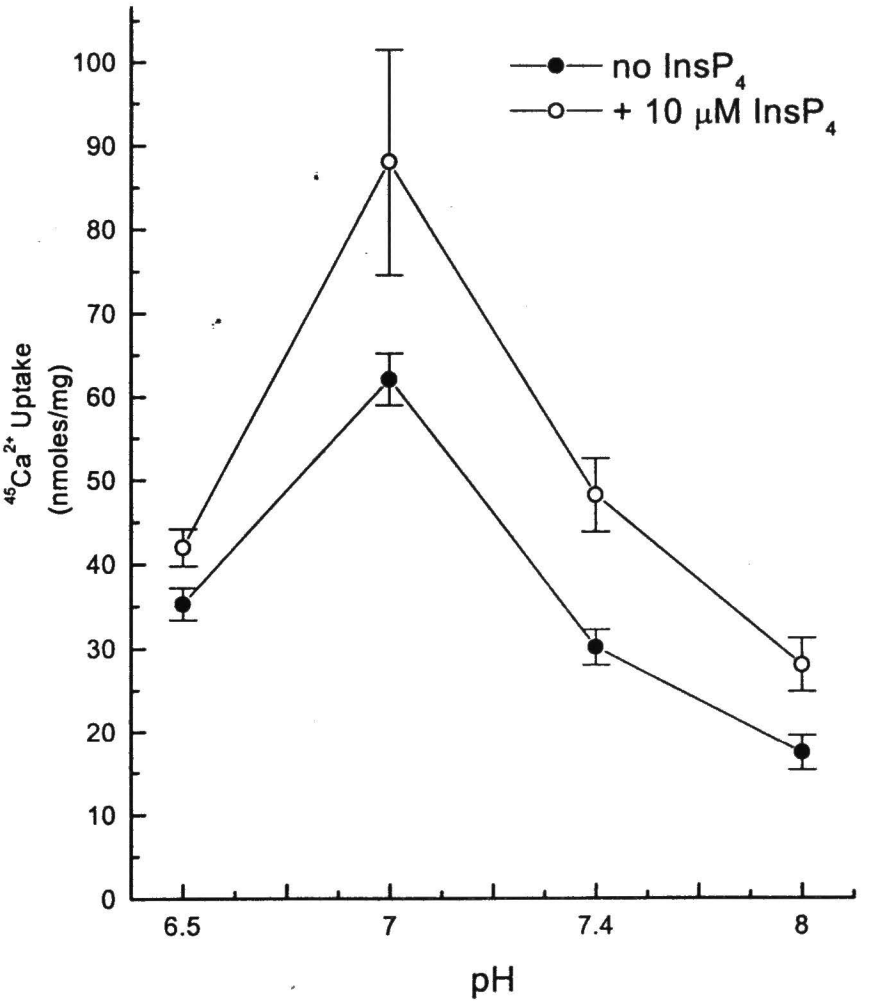

control
0.1 mM ATP
1 mM ATP
10 μM IP4
800
600
400
200
0
45Ca2+ Uptake
(nmoles/mg)
KCl control
60 mM NaCl

Fig. 12. pH effects on $InsP_4$ stimulated Ca^{2+} uptake in canine SL. A pH range between 6.5 and 8.0 was tested was tested on canine SL membranes to observe the effects of pH on $InsP_4$ stimulated Ca^{2+} transport. Uptake was performed at 30° C for 2 minutes.


no InsP4
+ 10 μM InsP4
45Ca2+ Uptake
(nmoles/mg)
100
90
80
70
60
50
40
30
20
10
0
6.5
7
7.4
8
pH

CHAPTER IV

DISCUSSION

There are a variety of different mechanisms to control Ca^{2+} levels in the heart. In the sarcolemma (SL) and transverse tubule (TT), there are L-type and T-type Ca^{2+} channels, a Na/Ca exchanger and various other inward Ca^{2+} currents including $i_{si,3}$ and leak channels (Lee et al., 1984; Benham and Tsien, 1987). The SL and associated TT are responsible for regulating Ca^{2+} flow across the outer membrane. It is through TT Ca^{2+} channels that Ca^{2+} flows into the cell during depolarization to cause Ca^{2+} induced-Ca^{2+} release. The control of Ca^{2+} across the SL is also critical for maintaining proper cardiac function. Of no less importance is the regulation of calcium inside the cell. Intracellular Ca^{2+} concentration is maintained at approximately 0.1 μM through regulation by proteins in the transverse tubules, sarcolemma and sarcoplasmic reticulum. The low resting intracellular calcium levels are essential for the relaxation of the myocyte. If levels of calcium were higher, a constant state of contraction would occur. Therefore, there are pools of calcium inside the cell as well as a high concentration of calcium in the extracellular fluid. The most notable intracellular storage site for calcium is the sarcoplasmic reticulum (SR). The SR is the major storage site for Ca^{2+} in the heart, although there are other related storage sites including calciosomes and possibly $InsP_3$ sensitive Ca^{2+} stores (Nosek et al., 1986; Kentish et al., 1990; Vites and Pappano, 1990).

During depolarization, Ca^{2+} quickly leaves these storage areas and allows for a contraction to occur. As mentioned above, removal of calcium from the intracellular space must occur quickly and efficiently. The SR-Ca-ATPase (SERCA) is an active transport mechanism located on the SR membrane. Other removal mechanisms include the Na/Ca exchanger and an SL Ca ATPase. Defining the role of the novel $InsP_4$ stimulated Ca^{2+} transporter requires some knowledge of the subcellular localization of this transporter.

Studies were done using different membrane preparations from either dog or cow. The $InsP_4$ stimulated Ca^{2+} transporter was found to be highest in membranes enriched with sarcolemma (Figs.1, 2, 4). These preparations included the SL preps and the 22.5% sucrose gradient membranes. This was true for both dog and cow. Although the $InsP_4$ stimulated Ca^{2+} transport was highest in SL derived fractions, there was some $InsP_4$ stimulated transport in microsomes and non SL derived vesicles. There are several explanations that may account for these results. First, the $InsP_4$ stimulated Ca^{2+} transporter may be present throughout the cell. This is supported by data from Huisamen et al. (1996) who found $InsP_4$ binding to both internal and external membranes. A more likely explanation, however, is that the isolation techniques used to prepare the membranes could not completely dissociate the internal and external membranes. Several internal structures of the cell are in close proximity to the outer membrane. These include the SR, Golgi and other storage compartments in the cell. It is entirely possible that the connections between the two membrane types were not broken during homogenization

leading to cross-contamination of SL and internal membranes. In fact, SL specific markers such as Na/K ATPase activity were present throughout all of the preps (Fig. 4), however, the relative activity between the preps were higher in those derived from the SL fractions.

Another challenge has been detection of TT versus SL vesicles. My original thought was that $InsP_4$ was stimulating a Ca^{2+} transporter in the transverse tubules. This would make the most sense, because entry of calcium through the TT triggers calcium induced calcium release, and stimulation of the $InsP_4$ stimulated transporter could aid in that process. However, due to a variety of problems, including no definitive marker for TT, this theory has not been proven. Future studies may focus on using novel techniques to differentiate the location of the $InsP_4$ stimulated Ca^{2+} transporter from SL derived fractions.

In Fig.3, canine 22.5% gradient vesicles were assayed under phase I conditions (see methods). One notable difference between phase I and phase II experiments is the decreased calcium uptake in phase I reactions. Typically, phase I reactions have less than half the Ca^{2+} uptake values as compared to phase II reactions (Figs. 6a vs. 6b). In figure 3, $InsP_4$ gave a two fold increase in Ca^{2+} uptake as compared to the unstimulated uptake (background). $InsP_4$ stimulated Ca^{2+} uptake was not diminished by the addition of thapsigargin (5 μM), a known SERCA inhibitor, indicating that $InsP_4$ is not acting upon SERCA. When $InsP_4$ (10 μM) was tested with ATP (1 mM), there was no additivity of

Ca^{2+} uptake. At first, I interpreted this to mean that $InsP_4$ and ATP were loading into the same compartment and that compartment was already filled. However, the next point examined the combination of $InsP_4$ (10 μM), ATP (1 mM) and TG (5 μM). The uptake was reduced to background levels. This was in direct conflict with what I had expected. If TG only acted upon ATP dependent uptake and had no effect on $InsP_4$ stimulated uptake, then the uptake in the presence of all three should be equivalent to what $InsP_4$ would be alone. Since that was not the case, this lead to the conclusion that high levels of ATP may be having an inhibitory effect on $InsP_4$ stimulated Ca^{2+} transport.

The next undertaking was to determine what ions or nucleotides might regulate the $InsP_4$ stimulated Ca^{2+} transporter. There was already some evidence that ATP was important in regulating the $InsP_4$ stimulated Ca^{2+} transporter (Fig. 3), so with that, ATP concentration curves were done under a variety of conditions. Figures 5 and 6a represent ATP curves in bovine microsomal and SL preparations and canine 22.5% sucrose gradient membranes, respectively. It is important to note that the shape of the curve is very similar between the cow SL and microsomes (Fig. 5) and the dog 22.5% sucrose gradient membranes (Fig. 6a). In both species, these curves have been replicated on several occasions. The highest uptake on the ATP curve occurs between 0.1 and 0.3 mM. This may represent ATP stimulating the $InsP_4$ stimulated Ca^{2+} transporter. It is not likely to be SERCA uptake, because 22.5% sucrose gradient vesicles are low in SERCA uptake (Fig. 1). In addition, Ca^{2+} uptake that is stimulated by $InsP_4$ is maximal around 2 minutes (Figs. 8, 9, 10) while SERCA uptake is half maximal around 5 minutes. At higher

concentrations of ATP, Ca^{2+} uptake is inhibited (Fig 5, 6a). This may be due to the membrane becoming more permeable to Ca^{2+} ions or ATP acting upon Ca^{2+} channels. Since the $InsP_4$ stimulated Ca^{2+} transporter is driven by a calcium concentration, dissipation of that gradient will effectively shut down the ability of the transporter to transport calcium. Figure 6b represents a phase I ATP curve in canine 22.5% sucrose gradient vesicles. The values are lower in a phase I experiment as compared to a phase II (Fig. 6a vs. 6b), but the decrease in Ca^{2+} uptake is not as drastic. This may be because the increased protein concentration protects ATP dependent Ca^{2+} uptake. $InsP_4$ simulated Ca^{2+} transport is diminished at high ATP concentrations, although ATP dependent uptake may not be affected. In figure 4, although 1 mM ATP was able to illicit a response, $InsP_4$ was not able to in the presence of ATP. As discussed earlier, ATP dependent uptake by membranes may not be as affected by high ATP, in addition, this experiment was done using a phase I protocol. A paper by Kupriyanov (1983) suggests that ATP can increase the efflux of calcium across the sarcolemmal membrane of a cell. Due to the rapid loss of Ca^{2+} after 0.5 mM ATP, this seems like a plausible explanation. I have had much difficulty in obtaining consistent results using ATP concentrations of 0.5 mM or above under phase II conditions. High oxalate (10 mM) or high magnesium (5-10 mM) seems to be able to slow the loss of Ca^{2+} at high ATP concentrations. This may be due to blocking of openings in the membrane or changing binding characteristics of calcium in the cell.

Preparations high in SERCA seem to be less prone to loss of calcium at high ATP concentrations. In experiments using canine 40% vesicles or canine microsomes, the reduction in Ca^{2+} uptake was not as great as compared to predominately SL derived fractions. This may be because high ATP levels do not inhibit SR Ca^{2+} uptake or effect SR Ca^{2+} permeability. In an ATP concentration curve using dog microsomes, thapsigargin (5 μM) was added to some tubes along with 1 mM ATP. The reduction of calcium uptake was almost down to baseline (results not shown). Bovine microsomes do not appear to have an abundance of SR in them, as evidenced by the fact that ATP stimulated Ca^{2+} uptake is lower in bovine as compared to canine (Figs.1 and 6a).

Figure 7 shows the effects of different nucleotides on Ca^{2+} uptake. The results using ATP were difficult to obtain. A careful balance between Ca^{2+} concentration, membrane concentration, incubation time, ATP concentration and oxalate/magnesium had to be maintained for reliable results. The 0.33 mM ATP values are consistent with other experiments done under similar conditions. ATP is not enhancing $InsP_4$ stimulated Ca^{2+} uptake under these conditions. A weakness of this experiment was that the concentration of ATP used was high enough that it may have begun to cause inhibition of $InsP_4$ stimulated Ca^{2+} transport. However, to make comparisons with other nucleotides, a concentration had to be used that would be sufficient to observe the effects of other nucleotides. Neither GDP, nor AMP had any effect on Ca^{2+} uptake at 0.33 mM. This is consistent with other results obtained using mono- or di- phosphates. ADP was of particular interest. The basal Ca^{2+} uptake values when 0.33 mM ADP was added were

much higher than the control basal Ca^{2+} uptake levels. Even the $InsP_4$ stimulated Ca^{2+} uptake appeared to be increased, although other similar experiments have shown ADP to have little effect on the maximal $InsP_4$ stimulated Ca^{2+} uptake. It is not known at the present whether or not ADP is directly stimulating the $InsP_4$ stimulated Ca^{2+} transporter, or if it creates an high basal Ca^{2+} level through another mechanism.

Finding the requirements for optimal Ca^{2+} transport proved to be difficult. No particular ion seemed to be required for this transporter to function. This ruled out antiport or symport mechanisms, leaving the action of the transporter to be dependent upon the Ca^{2+} gradient itself. Of course, although not required, there were several ions that did impact the ability to transport Ca^{2+}. Increasing potassium ions seemed to increase the ability of the transporter to transport Ca^{2+}. Figure 8 shows a potassium chloride curve (balanced by choline chloride) in bovine microsomes. The unstimulated Ca^{2+} uptake rose slightly with increasing potassium concentration, but the 0.1 mM ATP stimulated curve tripled in value over it's 0 potassium value. In a similar experiment testing basal, ATP (0.1 mM) and $InsP_4$ (10 μM) ($InsP_4$ results shown on Fig. 8) stimulated uptake, the 0 potassium containing group was still able to increase Ca^{2+} uptake over basal levels by 1.25 and 2.5 times. Although potassium had been eliminated from that experiment, chloride ions were still present due to the fact ionic strength was balanced with choline chloride. Since chloride ions might have allowed the transporter to function, potassium sulfate was substituted for all ions with the exception of potassium. Because sulfate would not pass through the membrane, the only ion to consider was the

potassium ion which had already been shown to be unnecessary for Ca^{2+} transport. One of the major weaknesses of this experiment was the fact that the osmolality was not controlled for. Potassium sulfate, K_2SO_4, divides into three parts-two potassium and one sulfate. Unfortunately, I failed to consider that and therefore the control had 260 mOs while the potassium sulfate had 390 mOsm. The results appear to be believable, they agree with data from another lab member using rat microsomal membranes that did control for osmolality. Assuming that the experiment is still valid, the potassium sulfate was able to increase Ca^{2+} uptake by 4.5 fold when stimulated by 10 μM $InsP_4$, although the background and maximal uptake are greatly reduced as compared to the control. When 1 mM $MgCl_2$ was added into another potassium sulfate substituted group, the results were not significantly different. This implies that trace amounts of chloride, or magnesium, will not significantly alter Ca^{2+} uptake patterns.

Another substitution model used α-methyl-n-glucopyranoside, to substitute for all ions. It is important to note that there is an extremely small amount of Na^+ and Mg^{2+} ions present due to sodium azide, to knockout mitochondrial ATPase, and 3 mM magnesium sulfate. The concentration of ions present was less than 5 mM. The control was performed using HEPES buffer at pH 7.0 with 100 mM KCl, 20 mM choline chloride, 10 mM NaCl. The HEPES control increased by 89% over basal Ca^{2+} uptake when stimulated with 10 μM $InsP_4$. The n-methyl-α-glucopyranoside increased 41% over basal Ca^{2+} uptake when stimulated with 10 μM $InsP_4$. Again, this confirms that there is

no particular ion that is required for transport, however, ions do increase the activity of the $InsP_4$ stimulated Ca^{2+} transporter.

Sodium ions were tested for their ability to affect $InsP_4$ stimulated Ca^{2+} transport. The work done with sodium was done to determine that the vesicles were of sarcolemmal origin. The Na/Ca exchanger has been reported to be in the sarcolemma. Na/Ca exchange is a very fast acting, concentration dependent process. It is difficult to obtain consistent and reliable data using conditions suitable for Na/Ca exchange. Ca^{2+} concentration must be high and sodium must also be at a high enough level to allow for exchange to occur. Although some data showed what may have looked like Na/Ca exchange, the results were not very reproducible and I decided not to use them as evidence for determination of the location of the $InsP_4$ stimulated Ca^{2+} transporter. I did, however, use Na in several uptake experiments to determine if it was regulating the $InsP_4$ stimulated Ca^{2+} transporter. Figure 11 shows the results of an uptake experiment using potassium soup (normal conditions) or soup with 66 mM NaCl. The background Ca^{2+} uptake between the two groups is not significantly changed under control or 1 mM ATP conditions. Ca^{2+} uptake in the presence of 10 μM $InsP_4$ is slightly reduced , which may be an indication of Na/Ca exchange. Again, however, due to variability in results that may or may not be the case. Normal conditions did not include any significant amounts of sodium in the incubation or uptake media, concluding that sodium is not necessary for $InsP_4$ stimulated Ca^{2+} uptake to occur.

The effects of different pH on $InsP_4$ stimulated Ca^{2+} transport were examined. In order to contend that this is another possible calcium transporter, activity under normal physiological pH had to be shown. Using different buffers, a pH range between 6.5 and 8.0 was obtained. At a pH of 6.5, $InsP_4$ was able to elicit only a very small increase. Although the uptake was highest at pH 7.0, the percent increase by $InsP_4$ is smaller than at pH's of 7.4 and 8.0. Between pH 7.4 and 8.0, the percent $InsP_4$ stimulated Ca^{2+} uptake is the same, although the background is greatly reduced at pH 8.0. The increasing $InsP_4$ effect over this pH range suggests that there is a specific protein (or proteins) responsible for $InsP_4$ stimulated Ca^{2+} uptake and this protein is active at physiological pH.

Conclusions- The goal of this research was to determine the membrane location and ions or nucleotides that regulate the $InsP_4$ stimulated Ca^{2+} transporter. I believe that this goal has been accomplished. The $InsP_4$ stimulated Ca^{2+} transporter has been identified in SL membrane fractions from both the dog and cow. No particular ion is required for $InsP_4$ stimulated Ca^{2+} transport, however conditions with high potassium and low sodium are preferable. A pH between 7.0 and 8.0 allow $InsP_4$ stimulated Ca^{2+} transport to occur, with optimal uptake and stimulation occurring at pH 7.4. ATP concentrations for stimulation of the $InsP_4$ stimulated Ca^{2+} transporter should be less than 0.33 mM. Although under *in vitro* conditions ATP concentrations greater than 0.5 mM destroy $InsP_4$ stimulated Ca^{2+} transport, *in vivo* actions of ATP on the $InsP_4$ stimulated Ca^{2+} transporter are unknown. It is presumed, based on data from phase I and phase II data, as well as knowledge that ATP levels can reach high levels in a cell without cell

permeabilization and death, that high ATP will not destroy $InsP_4$ stimulated Ca^{2+} activity under *in vivo* conditions.

Due to the location of the $InsP_4$ stimulated Ca^{2+} transporter, it may act to increase Ca^{2+} entry across the sarcolemma. Although data is not conclusive on which area of the sarcolemma contains the $InsP_4$ stimulated Ca^{2+} transporter, the function is still likely to be Ca^{2+} entry across the membrane. $InsP_4$ is produced via an $InsP_3$/ $InsP_3$ kinase pathway. The time course for the formation of $InsP_4$ is in the order of minutes (Kohl et al., 1990) following stimulation of an $InsP_3$ coupled receptor. $InsP_4$ has been suggested as the agent responsible for the increased influx of Ca^{2+} following receptor activation (Scholz et al., 1992). If that action of the transporter was true, the transporter would be helpful in maintaining a positive inotropic effect, but what would the function be in the absence of receptor stimulation? In a quiescent cell, there would be no $InsP_4$ present, removing stimulatory effects on the $InsP_4$ stimulated Ca^{2+} transporter. Again, it is important to remember that the transporter is active without $InsP_4$, needing only a Ca^{2+} concentration gradient. Basal background levels of Ca^{2+} uptake may be due, in part, to the $InsP_4$ stimulated Ca^{2+} transporter. The Na/Ca exchanger transports sodium into the cell in exchange for calcium from the cell under resting conditions. To maintain the intracellular Ca^{2+} under resting conditions, calcium must be brought into the cell to balance the calcium lost through Na/Ca exchange and sequestration into intracellular compartments. Traditionally, leak channels have been proposed to accomplish the influx of Ca^{2+} under resting conditions, however, due to the large concentration gradient, the possibility of

transport into the cell by facilitated diffusion seems reasonable. This transporter may play a role in the maintenance of a Ca^{2+} gradient across the SL under resting conditions in addition to aiding in the positive inotropic effect of various stimulators.

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