CALCIUM TRANSPORT AND ATP HYDROLYTIC ACTIVITIES IN GUINEA-PIG PANCREATIC ACINAR PLASMA MEMBRANES

by

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ABSTRACT

The aim of the present investigation was to determine whether a plasma membrane high affinity Ca^{2+}-ATPase plays an integral role in the maintenance of cytoplasmic free Ca^{2+} in pancreatic acinar cells. To achieve this, the Ca^{2+}-transport and Ca^{2+}-ATPase activities were characterized and their properties compared. Plasma membranes from guinea-pig pancreatic acini were shown to contain an ATP-dependent high affinity Ca^{2+}-pump and a high affinity Ca^{2+}-dependent ATPase activity. In addition, a low affinity ATPase activity was also observed. The high affinity Ca^{2+}-ATPase activity as well as the Ca^{2+}-transport were found to be dependent on Mg^{2+}, whereas the low affinity ATPase activity appeared to be inhibited by Mg^{2+}. The high affinity ATPase activity was 7-fold greater in magnitude than the Ca^{2+}-transport. Whereas the Ca^{2+}-transport was very specific for ATP as a substrate, the high affinity Ca^{2+}-ATPase showed little specificity for various nucleotide triphosphates. These data would suggest that the Ca^{2+}-transport and the high affinity Ca^{2+}-dependent ATPase in guinea-pig pancreatic acinar plasma membranes may be two distinct activities

To further investigate whether the two activities were related, we investigated how the Ca^{2+}-transport and Ca^{2+}-ATPase activities were regulated by intracellular mediators. Regulation of the two activities by calmodulin, cyclic AMP-dependent protein kinase, Protein kinase C and inositol phosphates was investigated. Calmodulin failed to stimulate either activity. In addition, calmodulin antagonists, trifluoperazine and compound 48/80 produced a concentration-dependent inhibition of Ca^{2+}-transport. These data suggested the presence of endogenous calmodulin. Both antagonists failed to influence the Ca^{2+}-dependent ATPase activity. Experiments using boiled extracts from guinea-pig pancreatic acinar plasma membranes and erythrocyte plasma membranes Ca^{2+}-ATPase confirmed the presence of endogenous calmodulin.

Abstract

The catalytic subunit of cyclic AMP-dependent protein kinase stimulated Ca^{2+} transport, suggesting that cyclic AMP may have a role in the regulation of Ca^{2+}-pump-mediated Ca^{2+} efflux from pancreatic acini. Ca^{2+}-dependent ATPase activity, on the other hand, was not affected by the catalytic subunit. HA 1004, a specific inhibitor of cAMP-dependent protein kinase, failed to inhibit the Ca^{2+}-transport and Ca^{2+}-dependent ATPase activities. Since, this inhibitor was also ineffective at inhibiting the catalytic-subunit-stimulated Ca^{2+} transport, it may be concluded that HA 1004 is ineffective in blocking the actions of cAMP-dependent protein kinase in pancreatic acinar plasma membranes.

In our studies, purified protein kinase C, the phorbol ester TPA and the diacylglycerol derivative, SA-DG, failed to stimulate the Ca^{2+}-uptake activity. However, these agents produced stimulation of the Ca^{2+}-dependent ATPase activity in the presence of phosphatidylserine. CGP 41 251, a potent and selective inhibitor of protein kinase C, did not inhibit the Ca^{2+}-transport or Ca^{2+}-dependent ATPase activities. These observations suggest that protein kinase C may not be involved in the regulation of the plasma membrane Ca^{2+}-pump in guinea-pig pancreatic acinar cells. These results also point to another difference between Ca^{2+}-transport and the Ca^{2+}-ATPase activities in guinea-pig pancreatic acinar plasma membranes.

Neither inositol trisphosphate nor inositol tetrakisphosphate produced a statistically significant effect on Ca^{2+}-uptake, suggesting that IP_3- and/or IP_4-mediated Ca^{2+} releasing pathways may not operate in the isolated guinea-pig pancreatic acinar plasma membrane vesicles.

In summary, the results presented here provide evidence to suggest that the high affinity Ca^{2+}-ATPase is not the biochemical expression of plasma membrane Ca^{2+}-transport in panreatic acini. Our results imply a role for

calmodulin and cAMP-dependent protein kinase, but not protein kinase C, in the regulation of Ca^{2+} efflux from pancreatic acinar cells.

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ACh	acetylcholine
ADP	adenosine 5'-diphosphate
AP	ammonium persulphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
BAPTA	bis(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
^{o}C	degrees celcius
C-subunit	catalytic subunit of cAMP-dependent protein kinase
CaM	calmodulin
CaM-PK	calmodulin-dependent protein kinase
cAMP	adenosine 3',5'-cyclic monophosphate
CCh	carbachol
CCK	cholecystokinin-pancreozymin
CCK-8	CCK-octapeptide
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetate
cGMP	guanosine 3',5'-cyclic monophosphate
Ci	curie
cIP_3	cyclic 1:2,4,5-trisphosphate
CTP	cytosine 5'-triphosphate
DG	diacylglycerol
E_1	high affinity state of the enzyme
E_2	low affinity state of the enzyme
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum

GTP	guanosine 5'-triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
ITP	inosine 5'-triphosphate
K_{Ca}	concentration of free Ca^{2+} at half-maximal response
kDa	kilodaltons
KRB	Kreb's Ringer bicarbonate
LiDS	lithium dodecylsulphate
M	molar
μ	micro
mA	milliamperes
min	minutes
Mops	3-(N-morpholino)propanesulfonic acid
M_r	relative molecular mass
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced)
PA	phosphatidic acid
PC	phosphatidylcholine
PI	phosphatidylinositol
P_i	inorganic phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase (protein kinase A)
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
pNPP	*p*-nitrophenyl phosphate
PS	phosphatidylserine
RER	rough endoplasmic reticulum

rpm	revolutions per minute
S.E.M.	standard error of mean
SA-DG	1-stearoyl-2-arachidonoyl-*sn*-glycerol
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	tetraethyl-methylenediamine
TFP	trifluoperazine
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
Tris	tris(hydroxy)aminomethane
v/v	volume per unit volume
VIP	vasoactive intestinal polypeptide
V_{max}	maximal velocity
w/v	weight per unit volume
w/w	weight per unit weight
xg	unit gravitational force

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DEDICATION

To my parents for their continuous encouragement

INTRODUCTION

MORPHOLOGY AND FUNCTION OF THE EXOCRINE PANCREAS

Morphology

The pancreas is a solid glandular organ, structurally and functionally associated with the upper part of gastrointestinal tract (Harris et al., 1979). It has both endocrine and exocrine functions. The exocrine pancreas comprises the bulk of the organ. For example, 82% of pancreatic volume in guinea-pigs is exocrine tissue (Bolender, 1974).

Major structural components of the exocrine pancreas consist of acinar cells and the ductal cells. The acinar cells are organized into acini, which are small groups of cells arranged around a common luminal space. The acini are responsible for the synthesis and secretion of digestive enzymes. Ductal cells, on the other hand, are responsible for fluid and electrolyte secretions (Grossman and Ivy, 1946; Harper and Scratcherd, 1979).

The acinar cells are polarized cells consisting of basal, lateral and apical membrane surfaces (Fig. 1). Only the basal membrane is exposed to the circulation and contains receptors for secretagogues, while the enzymes are released at the apical membrane. The total cell volume is composed of 54% cytoplasmic matrix, 22% rough endoplasmic reticulum (RER), 8.3% nuclei, 8.1% mitochondria, 6.4% zymogen granules and 0.7% condensing vacuoles. The total membrane surface area consists of 60% RER, 21% mitochondria, 9.9% Golgi complex, 4.8% plasma membrane, 2.6% zymogen granules, 1.8% plasma membrane vesicles and 0.4% condensing vacuoles (Bolender, 1974). RER occupies the infranuclear and paranuclear regions of the cell, whereas the apical pole of the cell contains the Golgi complex and zymogen granules (Ekholm and

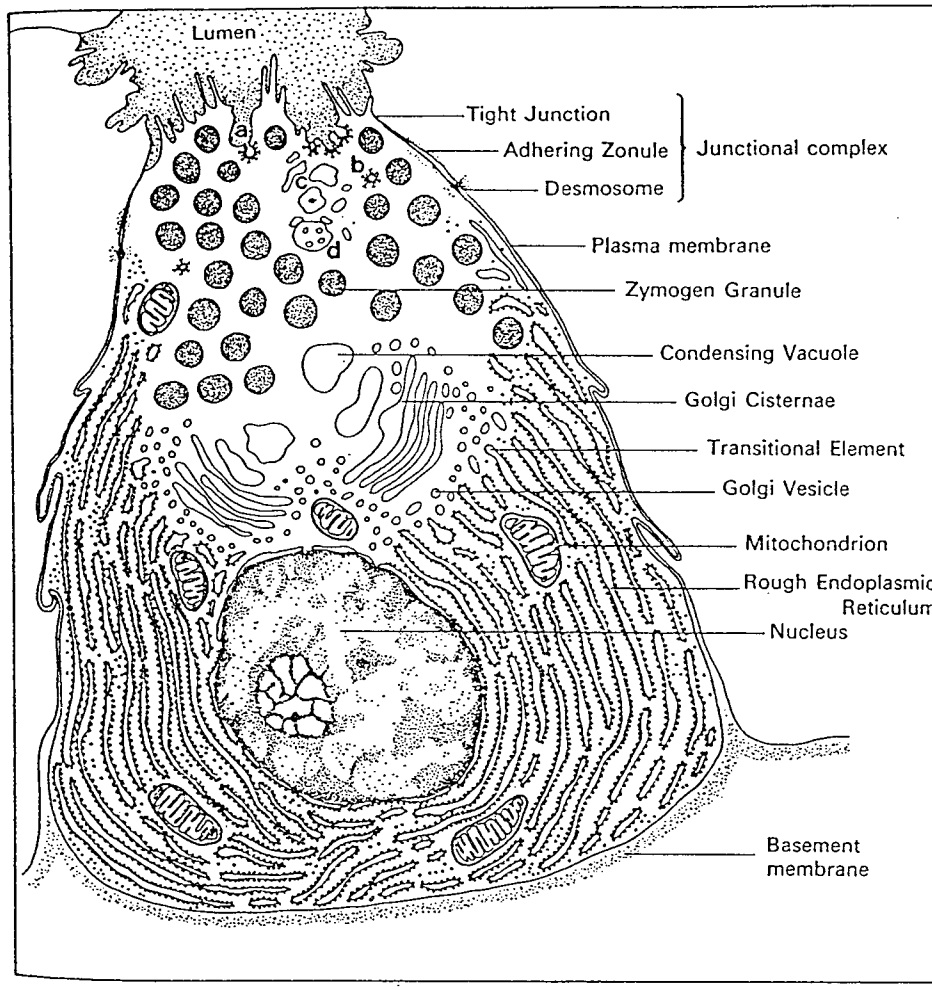

Figure 1. Diagramatic representation of a pancreatic acinar cell. In general, features of the resting cell are represented. However, the sequence of events (*a-d*) illustrated in the apical zone of the cell represent features of the stimulated cell. *a* = caveolus, *b* = coated vesicle, *c* = endocytotic vesicle, *d* = multivesicular body. (from Case, 1978).

Edlund, 1959; Ekholm et al., 1962a; Kern, 1986). Zymogen granules are the secretory units and are concentrated beneath the luminal plasma membrane to be released during stimulation.

In contrast to the acinar cell, the ductal cell cytoplasm contain relatively few mitochondria and small amounts of Golgi complex and RER (Dixon, 1979; Ekholm et al., 1962b). The ductal cells collectively form the duct system which is subdivided into three branches, the intralobular, interlobular and main pancreatic ducts (Bencosme and Lechago, 1969; Kern, 1986). These ducts perform the collecting and draining function of the pancreatic secretions. The intralobular ducts are located within the acinar lobules and drain the pancreatic juice secreted by the acini. Fluid from these ducts is drained into the interlobular ducts. These in turn drain into the main pancreatic duct which empties into the duodenum.

The Enzyme Secretory Process

Acinar cell secretion consists of a series of events including the synthesis of secretory product, its transport though intracellular compartments and its eventual release into the extracellular space. Most of our knowledge of the secretory pathway comes from the original studies by Jamieson and Palade (Jamieson and Palade, 1967, 1971a, 1971b, 1977, Palade, 1975). Their results can be summarized as follows: Secretory proteins are synthesized on RER and translocated into its cisternae. From here, the proteins are transported to the Golgi apparatus in small vesicles which are assumed to function as shuttling vesicles between the transitional elements of the RER and Golgi elements. After various modifications of the protein mixture within the Golgi compartments, the products are packaged within membranes derived from Golgi saccules. The proteins in the resulting condensing vacuoles are then progressively

concentrated to form the mature zymogen granules. These granules are stored just inside the apical membrane. An appropriate stimulation then leads to the fusion of zymogen granules with the apical plasma membrane and extrusion of their contents into the acinar lumen by a complex process of exocytosis (Fig. 2). This process is not well understood, but it appears to involve a specific and as yet unknown "recognition step" between the granule membrane and the apical plasma membrane, followed by the transient fusion of these membranes. After mediating the discharge process, the membrane material is recycled (Herzog and Reggio, 1980).

Composition of Pancreatic Acinar Cell Secretion

The human pancreas synthesizes and secretes more, as well as a wider range of proteins per gram of tissue than any other organ (Rinderknecht, 1986). However, because human tissue is, for the most part unavailable for study, much of our knowledge of pancreatic secretory proteins has been gained from studies in experimental animals.

Most of the early methods for separation of pancreatic secretory proteins involved column chromatography. However, in 1975, Scheele used two-dimensional electrophoresis to separate the mixture of proteins from guinea-pig exocrine pancreatic secretion (Scheele, 1975). This method yields higher resolution from smaller quantities of protein than the chromatographic separation methods. The secreted enzymes include serine proteases (such as trypsin, chymotrypsin and elastase), exopeptidases (including carboxypeptidases A and B), phospholipase A_2, lipase, colipase, nonspecific carboxylesterase, amylase, pancreatic ribonuclease and pancreatic deoxyribonuclease (Rinderknecht, 1986). A number of these enzymes are

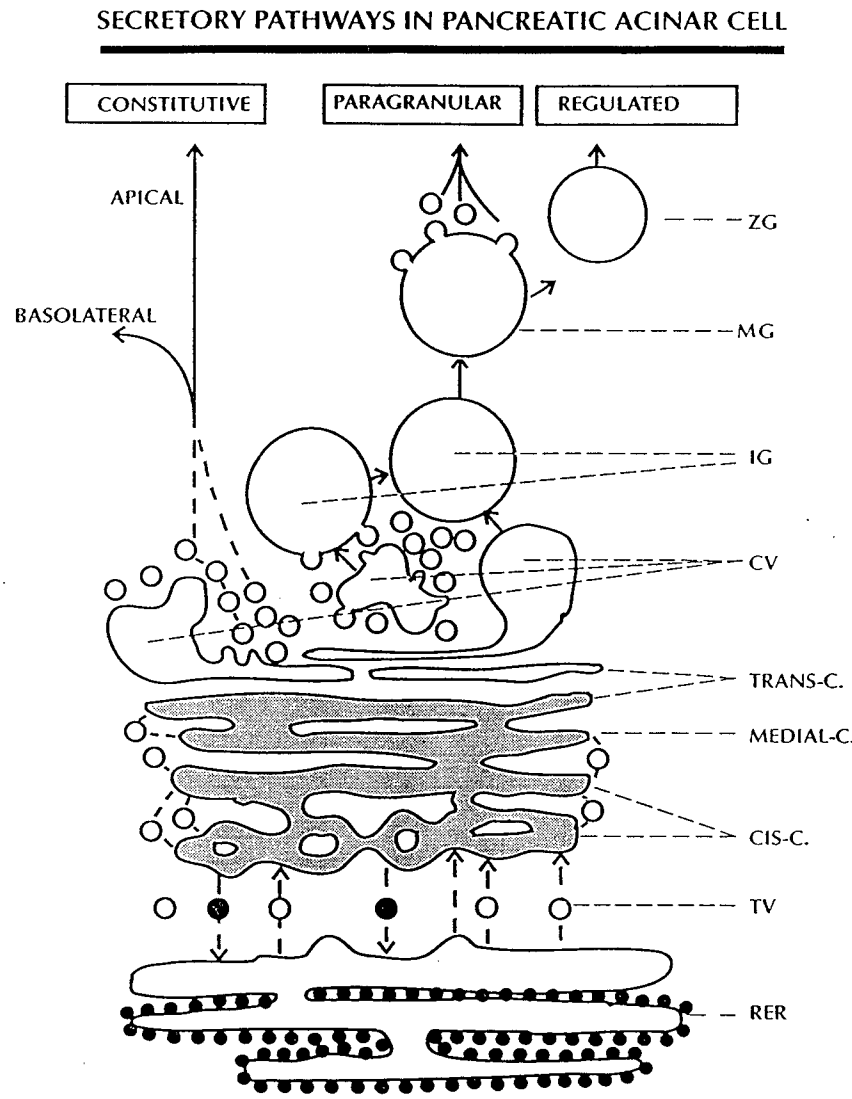

Figure 2. Scheme of the possible routes followed by secretory proteins in pancreatic acinar cells. The different organelles involved are shown. RER-rough endoplasmic reticulum, TV-transition vesicles, CV-condensing vacuoles, IG-immature granules, MG-mature granules, ZG-zymogen granules, C. (as in CIS-C.)-cisternae. (from Beaudoin and Grondin, 1991).

secreted as inactive zymogens that are activated by trypsin. Trypsin itself is converted to the active form by enterokinase secreted by the duodenal mucosa.

Regulation of Enzyme Secretion

Secretion of digestive enzymes from the exocrine pancreas is controlled by a number of peptide hormones and neurotransmitters present in the pancreatic ganglia (see Table I). However, the actual mechanism(s) involved in stimulus-secretion coupling are only now beginning to be understood. Cholecystokinin-pancreozymin (CCK) and the neurotransmitter acetylcholine (ACh) are known to participate in the physiological mechanisms for enzyme secretion. Evidence for the involvement of these secretagogues in enzyme secretion has come from both *in vivo* and *in vitro* studies.

The muscarinic cholinergic agents ACh, carbachol (CCh) and bethanechol can stimulate amylase secretion from pancreas (Argent et al., 1973; Case and Clausen, 1973; Williams and Chandler, 1975; Williams et al., 1976). Using radiolabelled antagonists, [^{3}H] quinuclidinyl benzilate and [^{3}H] N-methyl scopolamine, muscarinic cholinergic receptors have been demonstrated to be present on pancreatic acinar cells (Dehaye et al., 1984; Larose et al., 1981; Ng et al., 1979).

The other physiological stimulant, CCK, can stimulate amylase release two-fold in isolated pancreatic acinar cells (Christophe et al., 1976) and up to 20-fold in the perfused rat pancreas (Kondo and Schulz, 1976b; Williams, 1984). One possible explanation for this difference in amylase secretion is that exocytosis may require the presence of a specialized domain within the apical plasma membrane which is absent in isolated cells (Williams, 1984). The naturally occurring peptide analogues of CCK, gastrin and caerulein, were also

Table I. Regulators of pancreatic exocrine secretion and their intracellular messengers. (from Williams and Hootman, 1986).

Regulator	Intracellular messenger	Actions
ACh, CCK	Ca^{2+}	Digestive enzyme secretion; Cl^--rich pancreatic juice; digestive enzyme synthesis; trophic effects
Bombesin, Substance P	Ca^{2+}	Digestive enzyme secretion
VIP, Secretin	Cyclic AMP	Stimulation or potentiation of digestive enzyme secretion; pancreatic juice secretion
Insulin, epidermal growth factor	Unknown	Potentiation of digestive enzyme secretion
Somatostatin	Unknown	Inhibition of secretion

shown to increase enzyme secretion (Deschodt-Lanckman et al., 1976; May et al., 1978). Radiolabelled CCK (^{125}I-CCK) and other analogues have been used to demonstrate CCK binding sites on pancreatic acinar cells (Jensen et al., 1980; Sankaran et al., 1980, 1982).

High affinity binding sites for bombesin and related compounds have also been demonstrated on pancreatic acinar cells (Jensen et al., 1978). These compounds act on receptor sites distinct from the CCK receptors to evoke enzyme secretion (Jensen et al., 1978; Petersen and Philpott, 1979). Substance P and its analogues have also been shown to evoke enzyme release by acting on specific receptors (Jensen and Gardner, 1979, 1981).

Secretin and vasoactive intestinal polypeptide (VIP), while mainly involved in fluid and electrolyte secretion by the exocrine pancreas (Case et al., 1980; Fölsch and Creutzfeldt, 1977; Komarov et al., 1939; Said and Mutt, 1977; Sewell and Young, 1975), also regulate enzyme secretion in some species. These compounds, along with the peptide histidine isoleucine occupy specific receptors linked to the adenylate cyclase second messenger system leading to increased enzyme secretion in guinea-pig and rat, but not in cat, dog and mouse (Jensen and Gardner, 1981; Jensen et al., 1981, 1983; Robberecht et al., 1977).

The physiological relevance of insulin receptors on the surface of pancreatic acini (Korc et al., 1978) is not certain. Insulin may be a potentiator of enzyme release, rather than a stimulant (Williams et al., 1981).

STIMULUS-SECRETION COUPLING

As described above, the acinar cells are polarized cells. Therefore, the need for an intracellular messenger may be more pronounced in these cells than others since the physiological signal has to be carried from the stimulation sites at the basal membrane to the the enzyme releasing sites at the apical

membrane. The role of intracellular messengers has been extensively studied in the pancreatic acinar cell. Some of the accumulated knowledge is discussed below.

Role of Calcium

Enzyme secretion has been postulated to be triggered by increased cytosolic free Ca^{2+}. The involvement of cytosolic free Ca^{2+} in the signalling process during pancreatic secretion has been extensively reviewed (Schulz, 1980; Sung and Williams, 1988; Williams, 1980). Most secretagogues discussed above have been shown to increase intracellular free Ca^{2+}. It is now established that this increase occurs as a result of release from intracellular stores as well as an increased permeability of the plasma membrane to Ca^{2+}.

The resting levels of intracellular free Ca^{2+} in pancreatic acinar cells have been reported to be 90-105 nM in mouse (Ochs et al., 1985; Powers et al., 1985), 100-160 nM in guinea-pig (Pandol et al., 1985a) and 120-125 nM in rat (Bruzzone et al., 1986; Muallem et al., 1988b). This concentration increases to over 1 μM within seconds in response to stimulation by secretagogues (Muallem et al., 1988b; Ochs et al., 1985; Pandol et al., 1985a; Powers et al., 1985). This increase, however, is transient (Muallem et al., 1988c; Ochs et al., 1985; Pandol et al., 1985b; Streb and Schulz, 1983).

The requirement of Ca^{2+} for enzyme secretion was demonstrated in early experiments using Ca^{2+}-free buffers and ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-containing media. These studies showed that while initial enzyme secretion was independent of extracellular Ca^{2+}, sustained release could only be maintained in its presence (Argent et al., 1973; Case and Clausen, 1973; Elmerl et al., 1974; Williams, 1980). More recently, it has been shown that CCh failed to produce full stimulation of

enzyme release in the presence of bis(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), an intracellular Ca^{2+} chelator (Dormer, 1984). Furthermore, amylase release from pancreatic acini suspended in Ca^{2+}-containing media could be initiated with the Ca^{2+} ionophore A23187 (Elmerl et al., 1974; Ponnappa and Williams, 1980).

A good correlation between an increase in free Ca^{2+} and enzyme release was shown in isolated pancreatic acini (Dormer, 1984; Pandol et al., 1985a; Powers et al., 1985); antagonists of the secretagogues inhibited both amylase release and the rise in cytosolic free Ca^{2+} (Powers et al., 1985). The threshold concentration of intracellular free Ca^{2+} required for amylase release in the mouse pancreatic acini was calculated to be 280 nM (Ochs et al., 1985). A stoichiometric relationship appears to exist between changes in intracellular free Ca^{2+} and amylase release (Ochs et al., 1985). It was reported that 1.2% of total amylase is released over a 30 minute period for each 100 nM increase in Ca^{2+}.

Extensive research has been carried out to determine the source of the increased intracellular Ca^{2+}. An early study demonstrated that in superfused pancreatic fragments, amylase release in response to short pulses of ACh stimulation at half-hour intervals was not affected by exposure to a Ca^{2+}-free solution, even when EGTA was present (Petersen and Ueda, 1977). This study and others have shown that Ca^{2+} may be released from an intracellular store. Secretagogues have been shown to increase both release of intracellular Ca^{2+} and its influx. The initial effect of secretagogues appears to be the release of Ca^{2+} from intracellular stores (Pandol et al., 1987). CCh caused a rapid rise in intracellular free Ca^{2+} in the presence or absence of extracellular Ca^{2+} in mouse (Muallem et al., 1988c) and guinea-pig pancreas (Pandol et al., 1985a, 1987). Similar results were obtained with CCK in guinea-pig pancreas (Pandol et al.,

1985a). After stimulation with CCh, the Ca^{2+} store appears to be depleted or desensitized to CCK (Pandol et al., 1987) indicating that both secretagogues release Ca^{2+} from a common intracellular store (Powers et al., 1985; Schulz, 1980).

The release of sequestered Ca^{2+} has also been proposed to be the cause of an initial increase in Ca^{2+} efflux (Dormer et al., 1981) as observed in many studies using $^{45}Ca^{2+}$ prelabelled acini (Case and Clausen, 1973; Heisler, 1974; Kondo and Schulz, 1976a; Matthews et al., 1973). Early evidence suggested mitochondria to be the intracellular Ca^{2+} store (Clemente and Meldolesi, 1975; Wakasugi et al., 1982). However, more recent evidence has shown that Ca^{2+} released during stimulation originates from the endoplasmic reticulum (ER) (evidence discussed in the next subsection). A recent report has suggested two separate non-mitochondrial intracellular pools (Thévenod et al., 1989).

In addition to their action in initiating the release of Ca^{2+} from intracellular Ca^{2+} stores, secretagogues have been reported to increase plasma membrane permeability to Ca^{2+} in pancreatic acinar cells (Wakasugi et al., 1981). Activation of rat pancreatic acini by CCK was shown to result in a 7-fold increased permeability of the plasma membrane to Ca^{2+} (Muallem et al., 1988b).

Direct uptake of $^{45}Ca^{2+}$ into dispersed acini can be demonstrated with both secretagogues and the Ca^{2+} ionophore A23187 (Kondo and Schulz, 1976b). The influx of extracellular Ca^{2+} appears to be more important in sustained release (Ochs et al., 1985; Schulz, 1980). As demonstrated in guinea-pig pancreatic acinar cells, the sustained phase of amylase release is unaffected by CCh-induced depletion of intracellular Ca^{2+} stores (Pandol et al., 1985a). However, using rapid time resolution, Ca^{2+} influx was shown to occur before internal release in some cells (Blackmore, 1988; Sage and Rink, 1987). It has also been suggested that low physiological concentrations of agonists cause Ca^{2+}

influx, while high concentrations produce Ca^{2+} release (Exton, 1988). Another study showed that the rate of rise in intracellular Ca^{2+} in response to CCh was dependent on extracellular Ca^{2+}, while the onset and peak amplitude were unaffected (Ochs et al., 1985). This indicates that, although not directly dependent on it, the rise in intracellular Ca^{2+} may be regulated by external Ca^{2+}.

Role of Inositol Phosphates

The physiological significance of agonist-stimulated breakdown of Phosphatidylinositol (PI) was first suggested by Michell (1975). The second messenger involved in the release of Ca^{2+} from intracellular stores is inositol-1,4,5-trisphosphate (IP_3). The receptor-mediated activation of phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) (Putney et al., 1983) to produce IP_3 (Berridge and Irvine, 1984; Doughney et al., 1987; Pandol et al., 1985b). Hydrolysis of PIP_2 to diacylglycerol (DG) and IP_3 has now been established as a part of the signal transduction mechanism in the control of a variety of cellular processes such as secretion, metabolism, phototransduction and cell proliferation (Berridge and Irvine, 1984). Whereas IP_3 is water soluble and is released into the cytosol to mobilize intracellular Ca^{2+}, DG operates within plasma membranes to activate protein kinase C (PKC). Over the past few years, evidence has also accumulated indicating the production of IP_3 from PIP_2 during receptor-mediated stimulation of amylase release from pancreatic acinar cells. Secretagogues such as CCh and caerulein have been demonstrated to cause PIP_2 breakdown in the exocrine pancreas (Putney et al., 1983). Following agonist stimulation, the increase in IP_3 is rapid (Powers et al., 1985). In other experiments, it was shown that CCK-octapeptide (CCK-8) stimulated PI and PIP_2 breakdown in guinea-pig pancreatic acini in the absence of both

extracellular and intracellular Ca^{2+} (Pandol et al., 1985b). This suggests that the phosphoinositide breakdown is independent of extracellular Ca^{2+} and mobilization of intracellular Ca^{2+} stores. The hydrolysis of phosphoinositides is not accelerated by A23187. In fact, PI turnover was shown to precede Ca^{2+} release (Putney et al., 1983). Furthermore, production of IP_3 is noted within 5 seconds of stimulation by CCh, which is rapid enough to cause the release of sufficient Ca^{2+} from RER to give rise to the observed increase in cytosolic free Ca^{2+} (Doughney et al., 1987). Another study showed that the increase in intracellular free Ca^{2+} is transient, whereas PI breakdown continues after 5 minutes (Pandol et al., 1985b).

There is evidence to suggest that IP_3 causes the release of Ca^{2+} from intracellular stores, possibly ER or calciosomes. IP_3 caused Ca^{2+} release from nonmitochondrial stores in permeabilized insulin-secreting cells (Biden et al., 1984). In other tissues, such as permeabilized smooth muscle cells, IP_3 has been shown to mimic the effects of receptor-linked agonist on both contraction and the mobilization of intracellular Ca^{2+} (Bitar et al., 1986). Streb and colleagues were the first investigators to show a direct release of Ca^{2+} from nonmitochondrial intracellular stores by IP_3, using permeabilized rat acinar cells (Streb et al., 1983). Another study demonstrated that CCh and IP_3 act on the same pool of releasable Ca^{2+} (Streb and Schulz, 1983). The nonmitochondrial store was the same Ca^{2+} store which is released by ACh. Using rat pancreatic acinar cells, this IP_3 releasable intracellular Ca^{2+} store was later demonstrated to be the ER (Streb and Schulz, 1983; Streb et al., 1984). Other reports have since confirmed these findings (Brown et al., 1987; Muallem et al., 1987; Richardson and Dormer, 1984).

IP_3-induced translocation of Ca^{2+} across the ER membrane was shown to be through an ion channel rather than a carrier (Joseph and Williamson, 1986).

This IP_3-activated Ca^{2+} channel is blocked by heparin (Ferris et al., 1989). The Ca^{2+} efflux across ER membranes was balanced by the movement of monovalent cations and anions in permeabilized hepatocytes (Joseph and Williamson, 1986). An IP_3 receptor has recently been solubilized, purified and characterized from rat cerebellum (Supattapone et al., 1988b). This protein has a molecular mass of 260,000 daltons and a K_d for IP_3 of 0.1 μM. The binding of IP_3 to the receptor was reversibly inhibited by 300 nM Ca^{2+} in the particulate fraction, but not affected by up to 1.5 mM Ca^{2+} in purified receptor preparations (Supattapone et al., 1988b). The IP_3 receptor has been localized to the ER (Ross et al., 1989), reconstituted (Ferris et al., 1989, 1990) and sequenced (Furuichi et al., 1989; Maeda et al., 1990).

A possible feedback inhibition mechanism may operate for Ca^{2+} release in pancreatic acinar cells: IP_3-mediated Ca^{2+} release has been shown to be inhibited by Ca^{2+} itself in permeabilized AR42J cells, a pancreatic acinar cell line (Willems et al., 1990; Zhao and Muallem, 1990). This inhibition was explained by a possible loosely attached cytosolic factor which interacts with the IP_3-sensitive Ca^{2+} channel in a Ca^{2+}-dependent manner to modulate the IP_3-induced Ca^{2+} release.

IP_3 has also been suggested to regulate Ca^{2+} movements across the plasma membrane. It stimulated Ca^{2+} influx into T-lymphocytes (Kuno and Gardner, 1987) and rat mast cells (Penner et al., 1988). IP_3 also inhibited $^{45}Ca^{2+}$ efflux from rat brain synaptosomes (Fraser and Sarnacki, 1990). However, in other studies, IP_3-induced Ca^{2+} release from subcellular fractions of rat pancreatic acini did not correlate with plasma membrane markers (Streb et al., 1984).

Cyclic 1:2,4,5-trisphosphate (cIP_3), produced during agonist stimulation, has also been shown to release sequestered Ca^{2+} with the same potency as IP_3

(Irvine et al., 1986; Wilson et al., 1985). However, the turnover rate of cIP_3 is considerably slower than that of IP_3 itself. Therefore, the cIP_3 formed probably would not directly influence either Ca^{2+} release or Ca^{2+} entry. However, since IP_3 is short-lived, cIP_3 may take over the role of IP_3 such that the rapid metabolism of the latter is no longer of consequence (Hughes et al., 1988).

Inositol 1,3,4,5-tetrakisphosphate (IP_4) has also been shown to rapidly increase in pancreatic acinar cells during stimulation and has been suggested to increase Ca^{2+} entry into these cells (Muallem, 1989). There is direct evidence for the role of IP_4 in the stimulation of Ca^{2+} influx in sea urchin oocytes (Berridge, 1987). IP_4 may initiate Ca^{2+} signals by opening voltage-sensitive Ca^{2+} channels in the plasma membrane (Petersen, 1989). Binding sites for IP_4 have been demonstrated in many cells including the adrenal cortex (Enyedi and Williams, 1988). Other reports have suggested that IP_4 is not involved in the regulation of extracellular Ca^{2+} entry (Hill and Boynton, 1990). In perfused lacrimal glands, it was shown that the actions of ACh on Ca^{2+} entry could only be mimicked in the presence of both IP_3 and IP_4 and not by either agent alone (Morris et al., 1987). IP_4 was recently postulated to be involved in replenishing the IP_3-sensitive Ca^{2+} pool, possibly via interaction with the Ca^{2+}-ATPase of ER or calciosomes rather than the plasma membrane (Hill and Boynton, 1990). This might be explained by an interesting model proposed by Irvine (1990) in which the IP_4 receptor is located between ER and the plasma membrane when the two membranes are in close apposition.

Involvement of Protein Kinase C

Evidence has suggested that a rise in intracellular Ca^{2+} by itself is not sufficient to stimulate and maintain secretion from isolated acini (Sung and Williams, 1988). For example, the Ca^{2+} ionophore ionomycin produced only one-

third the level of amylase release compared to CCh in mouse pancreatic acini (Ochs et al., 1985), and CCh was shown to produce a 3-fold higher effect than maximal Ca^{2+} concentrations in permeabilized cells (Kimura et al., 1986). Furthermore, a divergence between Quin-2 fluorescence changes and amylase secretion has been observed at supramaximal agonist concentrations (Ochs et al., 1985; Powers et al., 1985). These data suggest that an additional event occurs at higher agonist concentrations. This additional stimulatory pathway may involve PKC. An endogenous PKC activity has been identified in the pancreas (Burnham and Williams, 1984; Wrenn et al., 1981). DG, the other product of PIP_2 hydrolysis and the natural activator of PKC (Nishizuka, 1984, 1988), stimulated amylase secretion, but produced no change in intracellular Ca^{2+} in guinea-pig pancreatic acini (Pandol et al., 1985a). The phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), an activator of PKC, has also been shown to stimulate amylase secretion from guinea-pig pancreatic acini without affecting the intracellular free Ca^{2+} concentration (Ansah et al., 1986; Pandol et al., 1985a). In addition, synthetic 1,2 DG and other phorbol esters were shown to stimulate amylase release from pancreatic acini (Gunther, 1981; Merritt and Rubin, 1985).

Since the rise in DG is slow and prolonged compared to IP_3 (Exton, 1987), PKC has been proposed to affect the sustained release of enzymes rather than the initial secretion (Sung and Williams, 1988). Furthermore, PKC itself is phosphorylated and therefore can remain active after DG and intracellular Ca^{2+} levels have decreased (Exton, 1987). It has been postulated that the initial and sustained phases of secretion are mediated by two separate intracellular pathways, the initial phase being controlled by IP_3 and the sustained phase by DG (Pandol et al., 1985a).

PKC may also have a negative feedback effect over secretagogue-induced Ca^{2+} transients (Nishizuka, 1988). Preincubation of guinea-pig pancreatic acini with TPA produced a time- and dose-dependent inhibition (IC_{50} = 30 nM) of the CCh-induced increase in intracellular free Ca^{2+}, reaching maximal inhibition within 3 minutes (Ansah et al., 1986). TPA was also shown to produce a marked decrease in CCK-8-evoked pancreatic secretory response in the anesthetised rat (Francis et al., 1990). This response was blocked by polymyxin B, an inhibitor of PKC. In human platelets, PKC was reported to cause initial secretion and aggregation, followed by negative feedback on receptor-mediated mobilization of intracellular Ca^{2+} and hydrolysis of PIP_2 (Zavoico et al., 1985). The evidence provided by these studies suggests that PKC may have a direct regulatory role over intracellular free Ca^{2+}. In other studies, PKC was shown to act synergistically with the Ca^{2+} ionophore (Merritt and Rubin, 1985). It has been postulated that complete activation of amylase release requires stimulation of both Ca^{2+}-dependent and PKC pathways (Merritt and Rubin, 1985; Pandol et al., 1985a).

It has recently been suggested that phosphatidylcholine (PC) breakdown may be more important than PI hydrolysis in the regulation of PKC and perhaps other cell functions. The generation of DG from PC is quantitatively greater than from PIP_2 and may be a major factor in the regulation of PKC (Exton, 1988). Another report has suggested two separate pathways for DG formation (Cockcroft et al., 1985). According to this hypothesis, in an early phase, DG is formed from PIP_2 and in a later phase, from phosphatidic acid (PA) which is formed from PI via phospholipase D.

Other Intracellular Messengers of Pancreatic Enzyme Secretion

Secretin and VIP are believed to stimulate amylase release via cyclic adenosine 3',5'-monophosphate (cAMP) (Gardner and Jensen, 1981). Both agonists have been shown to increase cAMP levels between 8- and 30-fold in dispersed acinar cells or in intact acini from the guinea-pig pancreas (Korman et al., 1980; Robberecht et al., 1976). A much larger increase can be observed in the presence of a cyclic nucleotide phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthene (Gardner et al., 1982). Furthermore, somatostatin, which inhibits amylase release *in vivo*, inhibited the increase in cAMP by VIP and secretin in guinea-pig pancreatic acini (Esteve et al., 1983). These observations indicate that, at least in guinea-pig pancreas, cAMP plays a role in stimulus-secretion coupling. In other species, such as mouse, cAMP-mediated secretagogues do not affect amylase release by themselves, but can potentiate the release by Ca^{2+}-mediated secretagogues (Burnham et al., 1988).

Another cyclic nucleotide, cyclic guanosine 3',5'-monophosphate (cGMP), is increased in response to Ca^{2+}-releasing secretagogues and A23187 (Christophe et al., 1976, Berridge, 1984). However, elevations of intracellular cGMP by addition of 8-bromo or dibutyryl cGMP produced no effect on Ca^{2+} fluxes or amylase release (Gunther and Jamieson, 1979). Therefore, the increase in cGMP levels may be secondary to increased Ca^{2+} via stimulation of guanylate cyclase. A potential mediator role for cGMP in stimulus-secretion coupling is not clear.

PA, which is formed during secretagogue-mediated phospholipid breakdown has been postulated to act as an endogenous ionophore to mediate the inward movement of Ca^{2+} that occurs during occupation of the surface membrane receptors (Putney et al., 1980).

Interactions Among Intracellular Messengers

The Ca^{2+}, PKC and cAMP-dependent protein kinase (PKA) systems appear to interact with one another to influence enzyme secretion. PKC and Ca^{2+} ionophore were shown to produce synergistic stimulation of amylase release in pancreatic acini (Kimura et al., 1986; Merritt and Rubin, 1985; Pandol et al., 1985a). PKA produced a stoichiometric phosphorylation of IP_3-binding protein in rat cerebellum to cause a substantial decrease in the potency of IP_3 in releasing Ca^{2+} (Supattapone et al., 1988a). Calmodulin-dependent protein kinase (CaM-PK) and PKC were found to be ineffective in phosphorylating this protein. On the other hand, in permeabilized rat pancreas, cAMP and analogues stimulated Ca^{2+}-induced secretion (Kimura et al., 1986). Furthermore, VIP, secretin and 8-bromo cAMP were shown to potentiate the effects of Ca^{2+}-mediating agents in the pancreas (Burnham et al., 1984; Collen et al., 1982). TPA has been shown to enhance the cAMP-mediated secretion (Kimura et al., 1986). This effect may be due to a PKC-induced phosphorylation of adenylate cyclase, leading to the potentiation of its activity (Yoshimasa et al., 1987). Adenylate cyclase activity can also be stimulated by CaM-PK (Klee and Newton, 1985). On the contrary, cAMP was shown to markedly inhibit Ca^{2+} mobilization and secretion in platelets (Nozawa, 1987). This effect may have been due to the stimulation of Ca^{2+} uptake into intracellular stores (Nozawa, 1987).

Intracellular Ca^{2+} Oscillations and Ca^{2+}-induced Ca^{2+} release

Recent developments have shown that instead of a continuous flow out of an open channel, Ca^{2+} may be released in 'quantal' form in response to secretagogue stimulation (Irvine, 1990, Muallem et al., 1989). This was probably best illustrated by an elaborate study performed in Xenopus oocytes using

confocal fluorescence Ca^{2+} monitoring to measure localized Ca^{2+} release by flash photolysis of caged IP_3 (Parker and Ivorra, 1990). This study demonstrated an abrupt onset of Ca^{2+} release in an all or none manner with increasing IP_3 liberation.

The quantal release of Ca^{2+} may explain the Ca^{2+} oscillations observed in single cell preparations. Yule and Gallacher (1988) noticed that the treatment of single pancreatic acinar cells with low concentrations of ACh produced oscillations of intracellular Ca^{2+}, detected by changes in fura-2 fluorescence. While these oscillations could be initiated in Ca^{2+}-free medium, they could only be sustained in Ca^{2+} containing medium. The frequency of these oscillations did not depend on the ACh concentration (Yule and Gallacher, 1988). The oscillations were more prominent at lower agonist doses (Stuenkel et al., 1989). On the other hand, low concentrations of CCK were shown to produce a small sustained release of Ca^{2+}, and Ca^{2+} oscillations were only observed at high CCK concentrations (Tsunoda et al., 1990).

It has recently been suggested that Ca^{2+} oscillations result from a small localized release of IP_3 (Zhao et al., 1990). If so, this may explain the results of Dormer and colleagues who demonstrated that inositol phosphate formation was less sensitive to CCh than was the stimulation of amylase release (Doughney et al., 1987). It is likely that these authors were unable to measure such a minute local release of IP_3. Ca^{2+} oscillations appear to die away during agonist application making way to a more sustained elevation of Ca^{2+} (Parker and Ivorra, 1990). This phenomenon probably reflects diffusion of Ca^{2+} across the cell and not IP_3 accumulation, since a recent study showed that a constant IP_3 infusion caused a steady release of Ca^{2+} resulting in repetitive Ca^{2+} spikes (Wakui et al., 1990). In fact, a constant supply of IP_3 appears to be necessary for Ca^{2+} oscillations. It is now established that activation of receptors linked to IP_3

formation generally evoke oscillating cytoplasmic Ca^{2+} signals with submaximal agonist concentrations (Goldbeter et al., 1990).

Although the mechanism for intracellular free Ca^{2+} oscillations in non-excitable cells is not clear, several suggestions have been made. It is possible that more than one mechanism exists (Zhao and Muallem, 1990). Studies with AR42J pancreatic cells indicate that Ca^{2+} oscillations result from a combination of stimulation of Ca^{2+} release by IP_3 and inhibition of Ca^{2+} induced Ca^{2+} release (Zhao et al., 1990). Since Ca^{2+} oscillations can be induced by IP_3, its agonists or Ca^{2+} itself, it was concluded that Ca^{2+}-induced Ca^{2+} release is responsible for the oscillations (Osipchuk et al., 1990; Wakui and Petersen, 1990). The Ca^{2+} oscillations are not due to fluctuations in the levels of IP_3 (Wakui et al., 1989). A lack of inhibition of Ca^{2+}-induced Ca^{2+} release by heparin indicates that it is not dependent on IP_3 or the IP_3-sensitive channel (Wakui et al., 1990). According to a recent study in single mouse oocytes, the role of IP_3-induced Ca^{2+} release may be to gradually raise Ca^{2+} levels to the point where Ca^{2+}-induced Ca^{2+} release is triggered (Peres, 1990).

The PKC pathway does not appear to be crucial for Ca^{2+} oscillations (Zhao et al., 1990), but it may have a negative feedback influence (Tsunoda et al., 1990). TPA was shown to inhibit Ca^{2+} oscillations triggered by a CCK analogue in AR42J cells (Zhao et al., 1990). It was proposed that the inhibitory regulation is due to the phosphorylation of a target protein by PKC. cAMP was also shown to negatively regulate the Ca^{2+} oscillations. The reduced amplitude and increased frequency of Ca^{2+} oscillations caused by cAMP (Zhao et al., 1990) may be due to the phosphorylation of the IP_3 receptor (Supattapone et al., 1988a).

Effect of Calcium on Exocytosis

Very little is known about the mechanism by which Ca^{2+} brings about secretion in acinar and other secretory cells. Calmodulin (CaM) has been suggested as a regulatory factor in the secretagogue activation of intracellular Ca^{2+} release in pancreatic acinar cells (Chien and Warren, 1988). In other studies using an ^{125}I-CaM gel overlay technique, CaM was shown to bind to a 230,000 daltons protein of possible cytoskeletal origin (Ansah et al., 1984). Therefore, it was suggested that CaM regulates the secretory process by interacting with the cytoskeleton (Ansah et al., 1984).

The molecular mechanism underlying the actions of Ca^{2+} during exocytosis has been proposed to involve specific or multifunctional CaM-PK (Cohn et al., 1987; Exton, 1987). To this end, a multifunctional CaM-PK of 51,000 daltons has been identified and purified from rat pancreas (Cohn et al., 1987; Gorelick et al., 1983). Many secretory processes are thought to involve CaM-dependent phosphorylation of cytosolic and membrane proteins (Exton, 1987). However, the Ca^{2+}-CaM complex may affect this phosphorylation without the kinase, and furthermore, Ca^{2+} may affect it without CaM. CaM-PK is known to modulate neurotransmitter release in the brain by acting on synapsin I which results in an altered interaction between synaptic vesicles and the plasma membrane (Llinas et al., 1985). A similar mechanism may be involved in pancreatic acinar cells. It is postulated that Ca^{2+}-dependent phosphorylation of a yet-unknown specific regulatory protein is involved in secretion. Phosphorylation of a ribosomal S6 protein was shown to be stimulated by CCh and CCK in rat pancreas (Freedman and Jamieson, 1983).

However, dephosphorylation of two proteins of 21,000 and 20,500 daltons appears to correlate better than phosphorylation with the onset of secretion (Burnham and Williams, 1982). Dephosphorylation of these proteins could be

prevented by atropine over a similar time period as the atropine-induced inhibition of amylase release. A Ca^{2+}-activated, CaM-dependent protein phosphatase identified and characterized in mouse pancreatic acinar cytosol (Burnham, 1985) may be responsible for this dephosphorylation.

In addition, Ca^{2+} may be responsible for the fusion of zymogen granules with the apical plasma membrane (Milutinovic et al., 1977).

CALCIUM HOMEOSTASIS

A precise regulation of cytosolic free Ca^{2+} is critical for this ion to serve as a physiological signal in the secretory process of pancreatic acinar cells. Extracellular Ca^{2+} concentration is 10,000-fold higher than intracellular levels, and this leads to a continuous leakage of Ca^{2+} into the cell down its electrochemical gradient. In addition, the cytosolic Ca^{2+} rises very rapidly during stimulation. Therefore, to terminate the stimulus and to maintain an appropriate resting free Ca^{2+} level, the cell must have mechanism(s) to continuously sequester and extrude excess Ca^{2+}. In the pancreas, the endoplasmic reticulum (Richardson and Dormer, 1984; Streb and Schulz, 1983), mitochondria (Wakasugi et al., 1982) and plasma membrane (Bayerdörffer et al., 1985a, b; Kribben et al., 1983; Schulz et al., 1986a) have all been ascribed roles in Ca^{2+} regulation.

Although mitochondria and ER have both been suggested as organelles involved in the intracellular sequestration of Ca^{2+} (Streb and Schulz, 1983; Wakasugi et al., 1982), recent evidence (Muallem et al., 1987; Schulz et al., 1986b) indicates that the ER may play the more important role. An ATP-dependent Ca^{2+}-uptake with a K_{Ca} of 1.1 μM was demonstrated in mouse pancreatic microsomes (Ponnappa et al., 1981). However, in more recent studies of rat pancreatic acinar ER, much higher affinities for Ca^{2+} have been reported

for the Ca^{2+}-pump and the Ca^{2+}-ATPase activities (Brown et al., 1987; Muallem et al., 1987).

A number of studies have shown that the ER Ca^{2+}-pump is activated during stimulation of the pancreas by secretagogues. ER isolated from secretagogue-stimulated pancreatic acini from rat and mouse showed increased Ca^{2+} uptake (Ponnappa et al., 1981; Richardson and Dormer, 1984). In addition, ER isolated from CCh-stimulated rat pancreatic acini showed decreased Ca^{2+} content and increased Ca^{2+}-ATPase activity (Brown et al., 1987). Both of these effects were prevented after blockade with atropine. More recently, Muallem and colleagues demonstrated that secretagogues stimulated the Ca^{2+}-pump of an agonist-sensitive Ca^{2+} pool (believed to be the ER) in permeabilized pancreatic acinar cells (Muallem et al., 1987, 1988d); the apparent affinity of this Ca^{2+}-pump for Ca^{2+} was increased 3-fold by secretagogue stimulation, while the maximal velocity was raised 2-fold (Muallem et al., 1987). The ER Ca^{2+}-pump was shown to regulate free Ca^{2+} down to 0.4 μM (Streb and Schulz, 1983). From the evidence available to them, Dormer and colleagues suggested that the ER Ca^{2+}-pump was more sensitive to free Ca^{2+} than the plasma membrane extrusion systems (Dormer et al., 1987). It is possible that one component of the nonmitochondrial Ca^{2+} pool is the recently described 'calciosomes' (Volpe et al., 1988). These structures are similar to ER and are distributed throughout the cytoplasm and may be the targets for IP_3.

Since the capacity of intracellular organelles to sequester Ca^{2+} is limited, extrusion of the cation from the cell is a necessity. It is here that the plasma membrane plays a very important role in the maintenance of intracellular free Ca^{2+} levels (Carafoli et al., 1986). In many mammalian cell types, two plasma membrane Ca^{2+}-extrusion mechanisms have been identified; a Ca^{2+}-transporting ATPase pump and a Na^+/Ca^{2+}-exchanger.

The Na^+/Ca^{2+}-exchanger utilizes a Na^+ gradient established by the Na^+/K^+-ATPase pump to drive its exchange of Na^+ for intracellular Ca^{2+} (Schatzmann, 1985). It is generally regarded as a low affinity system (Slaughter et al., 1987, Rengasamy et al., 1987). The precise role of this system in Ca^{2+} homeostasis of non-excitable cells remains unclear. Bayerdörffer and colleagues (Bayerdörffer et al., 1985b) showed that unlike other tissues, the Na^+/Ca^{2+}-exchanger in rat pancreatic acinar cells appears to be a high-affinity Ca^{2+} extrusion system. A high affinity Na^+/Ca^{2+}-exchanger has also been reported in human small intestine basolateral membranes (Kikuchi et al., 1988). A more recent study of the Na^+/Ca^{2+}-exchanger in dispersed pancreatic acini did not determine its affinity for Ca^{2+} (Muallem et al., 1988a). This system may work side-by-side with the Ca^{2+}-ATPase to extrude Ca^{2+} from the cell. However, despite its high affinity, the Ca^{2+} extrusion rate of the exchanger appears to be very low at approximately 0.12 nmoles/mg/min (Bayerdörffer et al., 1985b). In addition, some reports have indicated that the Na^+/Ca^{2+}-exchanger is not significantly stimulated during secretagogue-activation in pancreatic acini (Muallem et al., 1988a, b). Therefore, the exchanger may play only a minor role in Ca^{2+} homeostasis, with most, if not all, Ca^{2+} efflux from pancreatic acinar cells being mediated by the plasma membrane Ca^{2+}-pump (Muallem et al., 1988a).

The plasma membrane Ca^{2+}-pump may be more important in regulation and maintenance of low intracellular free Ca^{2+} levels in pancreatic acinar cells, but its activity in isolated plasma membrane preparations has not been sufficiently well characterized to fully assess this possibility. In rat pancreatic acini, the net Ca^{2+} efflux is thought to be regulated by the plasma membrane Ca^{2+}-pump (Muallem et al., 1988a). The rate of Ca^{2+} efflux in pancreatic acinar cells is increased during stimulation by secretagogues (Case and Clausen, 1973;

Heisler, 1974; Kondo and Schulz, 1976a; Matthews et al., 1973; Muallem et al., 1988b). It has also been suggested that the pancreatic acinar plasma membrane Ca^{2+}-pump can regulate intracellular Ca^{2+} concentrations to the same or a lower level than that achieved by the ER (Schulz et al., 1986b). The properties of plasma membrane Ca^{2+}-pump are discussed below.

The Plasma Membrane Calcium Pump

The active transport of Ca^{2+} is an ATP-requiring process and is believed to be carried out by a Ca^{2+}-ATPase enzyme (Sarkadi and Tosteson, 1979; Schatzmann, 1975). The plasma membrane Ca^{2+}-ATPase has been well characterized in the erythrocyte (reviewed by Carafoli et al., 1986; Schatzmann, 1986) and in cardiac muscle (Caroni and Carafoli, 1981a; Caroni et al., 1983), and has been linked to a Ca^{2+}-translocating function. The erythrocyte Ca^{2+}-ATPase is considered the prototype, and many of the properties discussed here were first described for this enzyme.

A Mg^{2+}-dependent Ca^{2+}-stimulated ATPase in the plasma membranes of human erythrocytes was first demonstrated by Dunham and Glynn (1961). Subsequent to this, a Ca^{2+}-transport activity was reported in these membranes (Schatzmann, 1966). The two activities were subsequently shown to be closely coupled (Schatzmann and Vincenzi, 1969). The Ca^{2+}-ATPase displays a high affinity for Ca^{2+} (K_{Ca} = 0.5 μM) and can hydrolyze ATP at a rate of 150-500 nmoles/mg/sec (Carafoli et al., 1986). Protons have been suggested as the counter ions for the erythrocyte Ca^{2+}-pump (Niggli et al., 1982). The K_{Ca} for cardiac sarcolemmal Ca^{2+}-ATPase is 0.4 μM (Caroni et al., 1983). In the absence of CaM, the purified Ca^{2+}-ATPases appear to have much lower affinities for Ca^{2+} than the enzymes *in situ* (K_{Ca} = 10 μM for erythrocyte and 20 μM for sarcolemmal) (Caroni et al., 1983; Niggli et al., 1981).

The erythrocyte enzyme demonstrates a high substrate specificity for ATP (Sarkadi et al., 1979). Although Ca-ATP and free ATP have been suggested as the physiological substrate of the enzyme, Mg-ATP generally appears to be the true substrate (Enyedi et al., 1982; Penniston, 1982). The number of Ca^{2+} ions pumped per mole of ATP hydrolyzed has been the subject of much controversy. While it appears that the stoichiometry of the pump can vary between 1:1 and 2:1 (Ca^{2+}:ATP) depending upon the concentration of the effectors used in the assay system (Akyempon and Roufogalis, 1982; Larsen et al., 1981; Sarkadi, 1980), a 1:1 ratio is generally thought to reflect the physiological situation (Carafoli et al., 1986; Larsen et al., 1978).

The molecular weight of the monomeric erythrocyte plasma membrane Ca^{2+}-pump determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is approximately 138,000 daltons (Carafoli et al., 1986). However, the enzyme is believed to be present within the membrane in an oligomeric form. A dimeric arrangement has been suggested by azidocalmodulin-binding experiments (Hinds et al., 1982). Molecular weight determinations by a radiation-inactivation study revealed that the functional form of the Ca^{2+}-pumping ATPase within the native erythrocyte membrane is a dimer of 290,000 daltons (Minocherhomjee et al., 1983).

A recent publication described the isolation and sequencing of cDNAs coding for a plasma membrane Ca^{2+}-pump from a human teratoma library (Verma et al., 1988). Comparison of the cloned sequence with the sequences from the purified erythrocyte Ca^{2+}-ATPase showed 86% identical residues. The calculated molecular weight of the translated clone is very similar to the observed M_r for erythrocyte Ca^{2+}-ATPase. Contrary to expectations, the primary sequence showed little resemblence to another extensively studied and cloned Ca^{2+} transporter, the sarcoplasmic reticulum Ca^{2+}-pump (Brandl et al.,

1986; MacLennan et al., 1985; Verma et al., 1988). A close resemblence between the two proteins is only observed in one hydrophobic region including residues 885-905 (Verma et al., 1988). Other resemblences between the two Ca^{2+} transporters are in the regions that are generally conserved in ion transporters such as the ATP binding site in the ATPases.

Controlled trypsin proteolytic digestion of the purified erythrocyte Ca^{2+}-ATPase results in a number of small successive fragments of 90, 85, 81 and 76 kilodaltons (kDa) (Benaim et al., 1984; Zurini et al., 1984). All of these fragments retain the Ca^{2+}-stimulated ATPase activity, while the 90, 85 and 81 kDa fragments can also transport Ca^{2+} following reconstitution into liposomes (Benaim et al., 1984, 1986; Zurini et al., 1984). The proteolysis of the 85 kDa fragment to the 81 kDa cleavage product results in a loss of the CaM binding site with a concommitant persistent stimulation of Ca^{2+}-ATPase activity.

The high affinity Ca^{2+}-transporting ATPase of erythrocyte membranes forms a Ca^{2+}-dependent phosphorylated intermediate which is acid-stable, but very labile in the presence of hydroxylamine and alkalis (Katz and Blostein, 1975). This phosphorylated intermediate has a molecular weight of 135,000-145,000 daltons (Katz and Blostein, 1975; Knauf et al., 1974; Wolff et al., 1977) and forms part of the reaction cycle of the enzyme.

The reaction sequence proposed by Schatzmann (1985) is shown in Figure 3. The transport cycle begins with the binding of Ca^{2+} and ATP to the enzyme, resulting in the formation of a high affinity Ca^{2+}-dependent phosphoenzyme, E_1CaP (Katz and Blostein, 1975; Niggli et al., 1979b; Rega and Garrahan, 1975; Richards et al., 1978). The formation of this intermediate does not require Mg^{2+} (Rega and Garrahan, 1975), but is accelerated by the presence of this ion (Enyedi et al., 1980; Rega and Garrahan, 1978; Schatzman and Burgin, 1978). In the next step, E_1CaP is converted to a more reactive intermediate E_2CaP

which has a lower affinity for Ca^{2+} and is hydrolyzed rapidly to release P_i in the presence of Mg^{2+} (Garrhan and Rega, 1978; Katz and Blostein, 1975). During this step, Ca^{2+} is thought to be transported to the extracellular surface of the cell (Sarkadi, 1980). The next step is the hydrolysis of the phosphorylated intermediate to E_2 and the dissociation of Ca^{2+} (Rega and Garrahan, 1978). The final step in the cycle is the conversion of the E_2 state back to the E_1 state.

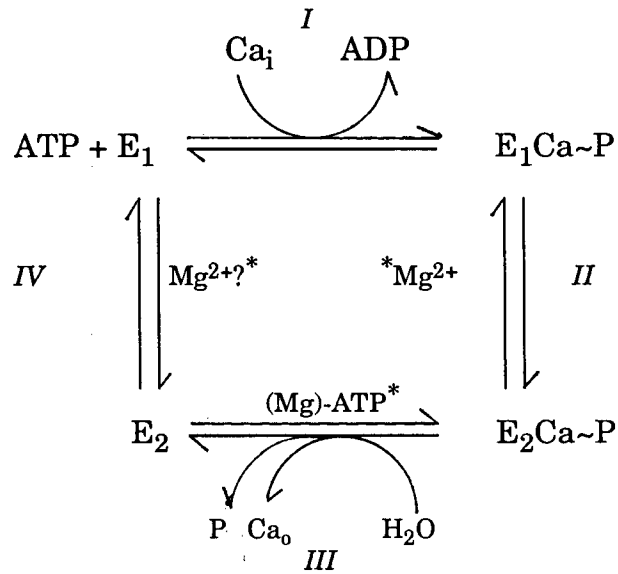

Figure 3. Reaction cycle of the human red cell Ca^{2+}-pump. In the normal mode the cycle turns in a clockwise direction. Ca_i represents ionized cytosolic Ca^{2+}, Ca_o represents ionized extracellular Ca^{2+} and P represents inorganic phosphate. E_1 and E_2 are two conformational forms of the protein. Requirements of the reactions are indicated by asterisks. (from Schatzmann, 1985).

A high affinity Ca^{2+}-ATPase has been demonstrated in rat pancreatic acinar cell plasma membranes (Al-Mutairy and Dormer, 1985; Ansah et al., 1984, Hurley et al., 1984). This activity shows characteristics similar to the Ca^{2+}-transporting ATPase of erythrocytes. It has a K_{Ca} of 0.65-1.7 μM (Al-Mutairy and Dormer, 1985; Ansah et al., 1984; Dormer and Al-Mutairy, 1987), shows an apparent requirement for Mg^{2+}, forms a Ca^{2+}-dependent

phosphorylated intermediate and is stimulated by CaM and acidic phospholipids (Ansah et al., 1984). In a recent study, a high affinity Mg^{2+}-dependent, Ca^{2+}-ATPase activity was localized to the cytoplasmic surface of the plasma membrane in rat pancreatic acinar cells (Ochs et al., 1988). In addition, a Ca^{2+}-transport activity has been observed in rat (Bayerdörffer et al., 1985a) and cat (Kribben et al., 1983) pancreatic acinar plasma membranes by one laboratory. The rat Ca^{2+}-pump was found to be electrogenic, had a K_{Ca} of 0.9 μM and was specific for ATP (Bayerdörffer et al., 1985a).

However, the results from most studies of pancreatic acinar plasma membranes demonstrate a low affinity, non-specific Ca^{2+}(or Mg^{2+})-ATPase activity, i.e. an enzyme which can only be stimulated with high concentrations of either Ca^{2+} or Mg^{2+}, and can utilize either di- or tri-phosphates as substrates (Forget and Heisler, 1976; Hamlyn and Senior, 1983; Lambert and Christophe, 1978; LeBel et al., 1980; Martin and Senior, 1980). Similar low affinity, Ca^{2+}(or Mg^{2+})-stimulated, di-/tri-phosphatase activities have been reported in a number of other tissue types such as rat stomach smooth muscle (Kwan and Kostka, 1984), corpus luteum (Minami and Penniston, 1987; Verma and Penniston, 1981) rat adipocytes (Pershadsingh and McDonald, 1980), cardiac muscle (Anand-Srivastava et al., 1982), liver (Lotersztajn et al., 1981, 1982), rat osteosarcoma (Murray et al., 1983) and rat kidney cortex (Parkinson and Redde, 1971). This enzyme appears to be insensitive to monovalent cations, ouabain, ruthenium red, vanadate and the mitochondrial inhibitors sodium azide and oligomycin.

The low affinity, non-specific Ca^{2+}(or Mg^{2+})-ATPase has been suggested to be an ecto-enzyme (Ansah et al., 1984; Hamlyn and Senior, 1983). The ecto-ATPase of pancreatic acinar cells (also referred to as a diphosphohydrolase) has been characterized and purified from the pig (LeBel et al., 1980). The purified

diphosphohydrolase can hydrolyze both di- and triphosphates with a pH optimum between 8 and 9. It is insensitive to oligomycin, ouabain and ruthenium red. The function of such ecto-enzymes is uncertain. Some reports suggest that in pancreas, it may be an enzyme located within the zymogen granule membrane which is inverted during exocytosis (Harper and Scratcherd, 1979); as such, it may be involved in the storage of zymogen granules and the process of exocytosis (LeBel and Beattie, 1985). Other functions for the ecto-ATPase have been postulated in other tissues. It was suggested that the ecto-ATPase of the mammary gland (13762 mammary-adenocarcinoma ascites), with similar properties to the pancreatic enzyme, may serve to control extracellular ATP concentrations (Carraway et al., 1980). An ecto-ATPase purified from rat heart sarcolemma is thought to play a role in the production of adenosine which increases coronary blood flow and the oxygen supply to the heart (Tuana and Dhalla, 1988). In mast cells, an ecto-ATPase was shown to be associated with Ca^{2+} influx (Chakravarty, 1987).

It is possible that two separate activities are present in the pancreas, a Ca^{2+}(or Mg^{2+})-ATPase and a distinct high affinity Ca^{2+}-ATPase that is responsible for Ca^{2+} transport. In addition to the low affinity Ca^{2+}(or Mg^{2+})-ATPase, a high affinity Ca^{2+}-ATPase activity has been demonstrated in some of the tissues mentioned above (Iwasa et al., 1982; Kikuchi et al., 1988; Lotersztajn et al., 1981, 1982; Minami and Penniston, 1987; Verma and Penniston, 1981). The high affinity Ca^{2+}-ATPase of liver plasma membranes has been suggested to be the Ca^{2+}-pumping protein (Lotersztajn et al., 1981; Pavoine et al., 1987). However, this activity differs from the erythrocyte prototype in a number of ways: it is not stimulated by CaM (Iwasa et al., 1982; Lotersztajn et al., 1981); it shows a higher Ca^{2+}-affinity (K_{Ca} = 13-87 nM); it has no requirement for external Mg^{2+} (Lin and Fain, 1984; Lotersztajn et al., 1981); it has a broader

nucleotide specificity; it has lower sensitivity to vanadate (Lin and Fain, 1984; Lotersztajn et al., 1981, 1982) and a stoichiometry of 0.3 (Ca^{2+}:ATP) for Ca^{2+} transport by the reconstituted purified ATPase (Pavoine et al., 1987).

Regulation of the Plasma Membrane Calcium Pump

Calmodulin

Calmodulin, as it was termed by Cheung (Cheung et al., 1978), is a ubiquitous protein (Cheung, 1980). It is a heat stable, acidic, Ca^{2+}-binding, globular protein with a molecular weight of 16,723 daltons (Klee and Vanaman, 1982). The highest concentration of CaM in mammalian tissues is found in the brain (Dedman et al., 1977; Watterson et al., 1976). CaM regulates a variety of cellular processes and stimulates a number of enzymes (Scharff, 1981). The first observation of the effects of CaM on Ca^{2+}-ATPase was made by Bond and Clough (1973), who reported that a non-hemoglobin protein present in the hemolysate of human erythrocyte membranes increased the Ca^{2+}-ATPase activity of isolated erythrocyte membranes. CaM has been demonstrated in and isolated from erythrocytes (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977). The stimulatory effects of CaM on Ca^{2+} transport and Ca^{2+}-ATPase in the plasma membrane vesicles from these cells are well established (Hinds et al., 1978; Larsen and Vincenzi, 1979; Penniston, 1983; Sarkadi, 1980; Schatzmann, 1982, 1985). This stimulation results in a 30-fold increase in the affinity for Ca^{2+} and 3-4 fold increase in maximum velocity of the pump (Foder and Scharff, 1981; Roufogalis and Mauldin, 1980; Scharff and Foder, 1978, 1982; Smallwood et al., 1988).

CaM generally requires Ca^{2+} for its actions. Saturation of CaM with Ca^{2+} results in a conformational change (Bromstrom and Wolff, 1981) which exposes

hydrophobic groups which in turn appear to be involved in the binding of CaM to the effector molecules (Tanaka and Hidaka, 1980). The suggested mechanism of CaM stimulation of Ca^{2+}-ATPase is the potentiation of both phosphorylation (Allen et al., 1987; Enyedi et al., 1980; Muallem and Karlish, 1980; Rega and Garrahan, 1980) and dephosphorylation (Allen et al., 1987; Jeffery et al., 1981; Rega and Garrahan, 1980) of the Ca^{2+}-dependent phosphoprotein intermediate, resulting in the increased turnover of the transport cycle.

CaM has also been isolated and purified to homogeneity from whole homogenates of dog pancreas (Bartelt et al., 1986). In addition, rat pancreatic acinar plasma membrane Ca^{2+}-ATPase can be stimulated by CaM (Ansah et al., 1984; Dormer and Al-Mutairy, 1987) and inhibited by micromolar concentrations of the CaM antagonists trifluoperazine (TFP) and chlorpromazine (Ansah et al., 1984). Furthermore, the ^{125}I-CaM gel overlay technique demonstrated Ca^{2+}-dependent binding of CaM to a 133,000 daltons protein which may be the Ca^{2+}-ATPase (Ansah et al., 1984). It should be noted, however, that the CaM-induced stimulation of Ca^{2+}-ATPases is not universal. The liver and corpus luteum Ca^{2+}-ATPases are not stimulated by exogenous CaM, even after washing the plasma membranes with EGTA to remove any endogenous CaM (Lotersztajn et al., 1981; Verma and Penniston, 1981); either CaM in these plasma membranes is very tightly bound or these enzymes are insensitive to it.

Oligomerization can stimulate purified Ca^{2+}-ATPase in the absence of CaM (Kosk-Kosicka et al., 1990). Since CaM inhibits oligomerization (Vorherr et al., 1991, Kosk-Kosicka et al., 1990), it was suggested that the CaM-binding domain may be involved in this process. Furthermore, a synthetic peptide CaM-binding domain appeared to bind the Ca^{2+}-ATPase (Enyedi et al., 1989; Vorherr

et al., 1991). These data implicate CaM-binding domain in the stimulation of the Ca^{2+}-pump.

Limited Proteolysis

Controlled tryptic digestion can also stimulate the high affinity Ca^{2+}-ATPase activity. Digestion of Ca^{2+}-ATPase to a fragment of 81,000 daltons results in stimulation of the enzyme to a similar extent as that observed with CaM treatment of the native enzyme (Benaim et al., 1984; Zurini et al., 1984). This cleavage also results in a loss of the CaM binding site. Therefore, it appears that the CaM binding domain exerts some type of inhibition on the Ca^{2+}-ATPase and that the masking of this domain by CaM-binding or oligomerization or its proteolytic cleavage removes this inhibition from the enzyme. Calpain, a Ca^{2+}-dependent endogenous protease (Pontremoli and Melloni, 1986) also stimulates Ca^{2+}-ATPase by proteolytic fragmentation (Wang et al., 1989). It too results in a loss of CaM stimulation, but yields different proteolytic fragments (Wang et al., 1988, 1989).

Other Regulators

Regulation of Ca^{2+}-ATPase by other agents has not been characterized to the same extent as the CaM and proteolytic regulation. The erythrocyte and cardiac sarcolemmal Ca^{2+}-ATPase are also stimulated by acidic phospholipids (Caroni et al., 1983; Niggli et al., 1981), and long chain fatty acids (Ronner et al., 1977). These enzymes are inhibited by vanadate (Caroni and Carafoli, 1981a; Niggli et al., 1981) and lanthanum (Caroni and Carafoli, 1981a; Schatzmann et al., 1986).

The Ca^{2+}-ATPase of cardiac sarcolemmal and erythrocyte membranes can also be stimulated by protein kinase A and protein kinase C. Ca^{2+}-transport in

plasma membrane vesicles from both sarcolemma (Caroni and Carafoli, 1981b; Neyes et al., 1985) and erythrocytes (Neyes et al., 1985) was stimulated by the cAMP-dependent protein kinase. However, there is disagreement over whether this kinase affects the rate of Ca^{2+} transport or the affinity for Ca^{2+}. The purified enzyme can be phosphorylated to form a hydroxylamine-resistant phosphoprotein in the presence of the catalytic subunit of PKA (Neyes et al., 1985).

In other studies, PKC in combination with TPA or diolein stimulated the Ca^{2+}-ATPase, Ca^{2+}-uptake and phosphorylation in erythrocyte plasma membranes (Smallwood et al., 1988). Similar findings were reported in smooth muscle cells, where the plasma membrane Ca^{2+}-pump could be stimulated by TPA (Furukawa et al., 1989). Studies in isolated pancreatic acinar cells using protein kinase inhibitors and TPA, have also indicated that PKC is required for activation of the Ca^{2+} pump (Muallem et al., 1988b). These reports suggest an involvement of PKC in the regulation of the Ca^{2+}-transporting ATPase.

As can be seen from the above discussion, our knowledge of the regulation of Ca^{2+} homeostasis within the pancreatic acinar cell is still incomplete.

OBJECTIVE

In erythrocytes and the cardiac sarcolemma, the role of the plasma membrane Ca^{2+}-ATPase in Ca^{2+} extrusion from the cell is well known. However, the existence of such a relationship between Ca^{2+}-ATPase and Ca^{2+}-transport within the pancreatic acinar cell has not been established. Most of the studies to date have concentrated on ATPase activity, while little information is available concerning the Ca^{2+} transport process within these cells.

In one study, a Mg^{2+}-dependent and Ca^{2+}-stimulated ATPase activity in the total particulate fraction of rat pancreatic acini was suggested to be the enzyme responsible for active Ca^{2+} translocation (Hurley et al., 1984). In another study, it was suggested that a Ca^{2+}(or Mg^{2+})-ATPase present in rat pancreas plasma membrane-rich fractions was unlikely to be involved in active Ca^{2+} extrusion (Forget and Heisler, 1976). To our knowledge, only one laboratory has been able to study Ca^{2+} transport in plasma membrane vesicles of pancreatic acinar cells (Bayerdörffer et al., 1985a; Kribben et al., 1983) and has proposed that this activity may be linked to a high affinity Ca^{2+}-ATPase.

To date there has not been a successful systematic attempt to characterize and correlate the Ca^{2+}-ATPase and Ca^{2+}-transport activities in pancreatic acinar cell plasma membranes. The principal reason for this difficulty appears to be the presence of masking Ca^{2+}(or Mg^{2+})-ATPase or diphosphohydrolase-like activities within these cells. The extent of the problem may vary depending upon the animal species chosen for the study.

Therefore, the aim of this study was to determine whether a high affinity Ca^{2+}-ATPase is responsible for Ca^{2+}-extrusion and hence Ca^{2+} homeostasis in pancreatic acinar cells. To achieve this, the high affinity Ca^{2+}-ATPase and Ca^{2+}-transport activities were characterized in the same plasma membrane preparations from guinea-pig pancreatic acini. These activities were compared

with respect to their requirement for Ca^{2+} and Mg^{2+}, their substrate specificity for different nucleotide triphosphates, and their regulation by various intracellular mediators such as protein kinase A, protein kinase C, CaM and inositol phosphates. In addition, an attempt was made to purify the high affinity Ca^{2+}-ATPase.

From the results of our studies, conclusions were drawn regarding the possible correlation of the high affinity Ca^{2+}-ATPase with the Ca^{2+} transport activity which could be assayed within our membrane preparations.

MATERIALS AND METHODS

MATERIALS

a). Radiochemicals:

γ-^{32}P-ATP (10-40 Ci/mmole) was purchased from Amersham Corporation (Oakville, ON) and ^{45}CaCl (10-40 mCi/mg calcium) was purchased from Amersham or New England Nuclear (Mississauga, ON).

b). Reagents:

The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO):

2-mercaptoethanol,

3-(N-Morpholino)propanesulfonic acid (Mops),

adenosine triphosphate (disodium),

adenosine triphosphate (tris),

ammonium bicarbonate,

aprotinin,

bromophenol blue,

calmodulin,

catalytic subunit of cyclic AMP-dependent protein kinase,

CDTA,

citric acid,

compound 48/80,

EDTA,

EGTA,

glucose,

glycerol,

glycine,

HEPES,

hydroxylamine,

Kodak GBX developer and replenisher,

Kodak GBX fixer and replenisher,

lithium dodecyl sulphate,

magnesium chloride,

β-nicotinamide adenine dinucleotide phosphate, reduced (NADPH),

polyethylene glycol,

silver nitrate,

sodium azide,

sodium bicarbonate,

Soybean trypsin inhibitor,

sucrose,

12-*O*-tetradecanoyl phorbol-13-acetate (TPA),

tetraethyl-methylenediamine (TEMED),

trichloroacetic acid (crystalline),

trifluoperazine,

Tris-base,

Tris-hydrochloride,

Triton X-100,

Tween 20.

The following chemicals were purchased from BDH Biochemicals (Toronto, ON): activated charcoal, calcium chloride, glacial acetic acid, methanol, potassium chloride, potassium phosphate (monobasic), sodium carbonate, sodium chloride, sodium dodecyl sulphate and sodium hydroxide.

Bovine serum albumin, dithiothreitol, inositol 1,3,4,5-tetrakisphosphate, inositol 1,4,5-trisphosphate and PMSF were purchased from Boehringer Mannheim Canada Ltd. (Laval, Québec).

The following electrophoresis chemicals were obtained from Bio-Rad Laboratories (Mississuaga, ON): acrylamide, N,N'-methylene-bis acrylamide, ammonium persulphate, Coomassie Brilliant Blue R-250 and high and low molecular weight standards.

The phospholipids, phosphatidylcholine, phosphatidylserine, and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SA-DG) were purchased from Serdary Research Laboratories (London, ON).

Formaldehyde, gluteraldehyde, and sodium phosphate (monobasic) were obtained from Fisher Scientific Co (Vancouver, B.C.).

Unisolve I® was purchased from Terochem Scientific (Edmonton, AB). HA 1004 was from Seikagaku Kogyo Co. (Japan), while CGP 41 251 and CGS 9343B were generous gifts from Ciba-Geigy Corporation (Summit, NJ). Fifty times concentrated amino acids containing L-glutamine were purchased from Gibco Laboratories (Burlington, ON) and collagenase (CLSPA) was obtained from Worthington Biochemical Corporation (Freehold, NJ).

METHODS

Preparation of Pancreatic Acini

Pancreatic acini from guinea-pigs were prepared by the method of Williams et al. (1978) with slight modifications. Pancreata from four guinea-pigs (250-300 g each) were isolated and placed into ice-cold Kreb's Ringer Bicarbonate (KRB), containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 14 mM Glucose, 0.1 mg/ml soybean trypsin inhibitor, amino acids and 2.5 mM $CaCl_2$ and equilibrated with 95% O_2/5% CO_2 for 20 minutes. The pH was adjusted to 7.35 with NaOH as required. Ten milliliters of dissociation solution (KRB containing 70-75 U/ml collagenase, 0.1 mM $CaCl_2$ and 0.1% bovine serum albumin) was then injected into the parenchyma of the pancreata. The organs were then transferred to a 50 ml conical flask and incubated for 15 minutes at 37°C in a water-bath oscillating at 120 cycles/min. After withdrawing the excess medium, 10 ml of fresh dissociation medium was added, followed by a further 45 minute incubation (as above). The acini were dissociated by drawing the tissue up and down through polypropylene pipettes having tip diameters of 1.5 mm and 0.9 mm. This and the following procedures were performed at room temperature. The dissociated tissue was filtered through a 150 micron Nytex® nylon mesh using an extra 20 ml of medium (without collagenase, but with 1% bovine serum albumin). The filtered acini were layered on KRB containing 4% bovine serum albumin and 0.5 mM $CaCl_2$ and centrifuged at 50x*g* for 4 min. The pelleted acini were washed twice with the same medium followed by another wash in the isolation medium containing 1.25 mM $CaCl_2$. Acini so prepared were used for isolating the plasma membranes as described below.

Preparation of Plasma Membranes from Pancreatic Acini

Plasma membranes were prepared by a modification of the method of Svoboda et al. (76). A flow-chart outlining the major steps in the preparation is shown in figure 4. All procedures were carried out at 4°C. The Acini were centrifuged at 500x*g*, and the pellet was suspended in 5 volumes 10 mM Tris-Cl, pH 7.4, containing 0.3 M Sucrose, 5 mM 2-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA). This suspension was subjected to glass-teflon, followed by glass-glass (Dounce) homogenization. The homogenate was diluted to 10 volumes and centrifuged at 180x*g* for 10 minutes. In some preparations, the resulting pellet was re-homogenized in a glass-glass homogenizer and centrifuged. The pooled supernatant was filtered through four layers of cheese cloth and centrifuged at 1,000x*g* for 10 minutes. The second supernatant was again filtered through cheese cloth and centrifuged at 150,000x*g* for 30 minutes. The pellet was suspended in the same buffer plus 2 mM EDTA, and layered on a discontinous sucrose density gradient consisting of 27%, 35% and 38% (w/w) sucrose layers. The tubes were centrifuged for 3 hours at 25,000 rpm in an SW28 swinging-bucket rotor (Beckman). The 27/35% interface, containing the plasma membranes, was collected and washed in sucrose-free and EDTA-free buffer. The final pellet was suspended in EDTA-free homogenization medium using a small glass-teflon homogenizer. The resulting plasma membrane vesicles were quick frozen and stored at -80°C until use.

Purification of Ca^{2+}-ATPase from Pancreatic Acinar Plasma Membranes

Ca^{2+}-ATPase from pancreatic acinar plasma membranes was purified by the method of Bridges and Katz (1986). The entire procedure was performed at 4°C. The pelleted plasma membranes were solubilized (unless otherwise stated)

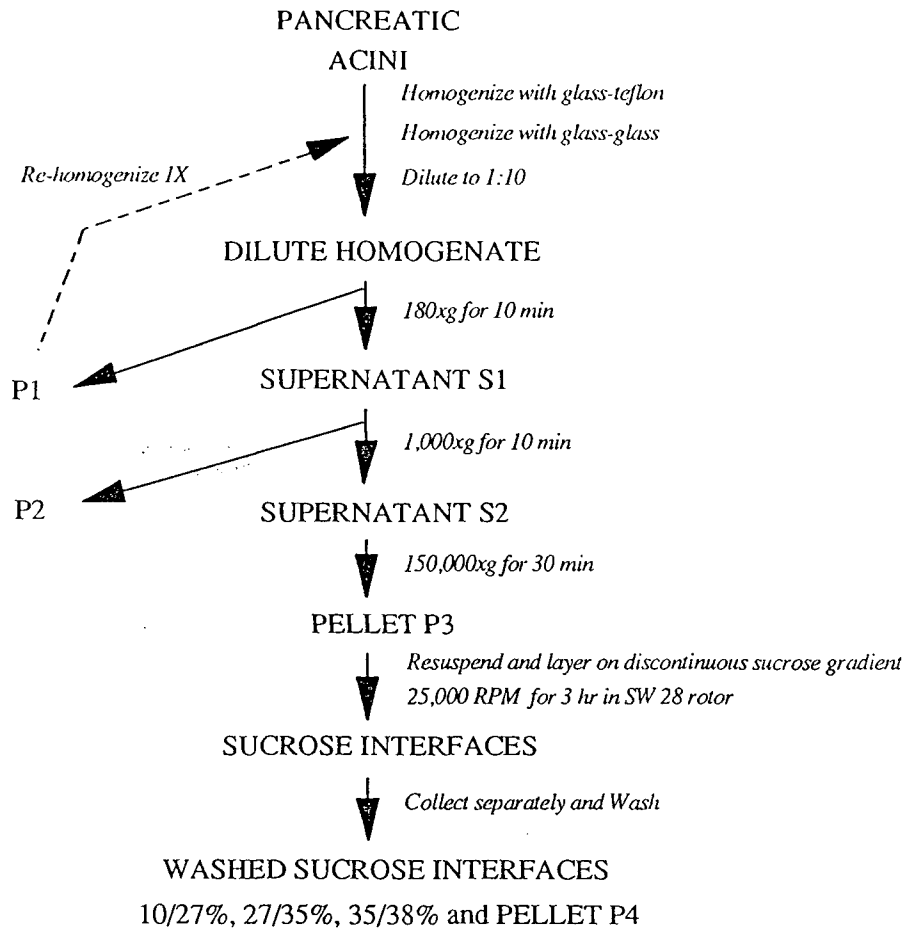

Figure 4. Flow-chart for the preparation of guinea-pig pancreatic acinar plasma membranes. Details are given in the text.

in a medium containing 10 mM HEPES, pH 7.4, 0.45% Triton X-100, 0.05% Tween 20, 300 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$ and 2 mM dithiothreitol. The solubilized membranes were centrifuged at 100,000x*g* for 30 minutes, and the supernatant was added to a CaM-agarose column pre-equilibrated with the solubilization buffer. Following binding, the column was washed with 25-100 bed volumes of Triton X-100-free solubilization buffer, and the bound protein was eluted with Ca^{2+}-free, EDTA (2 mM)-containing washing buffer. The chelator in the eluent was "neutralized" by 2 mM $CaCl_2$. The enzyme was either assayed fresh or quick frozen for storage at -80°C.

Measurement of Calcium Uptake Activity

ATP-dependent Ca^{2+}-transport into guinea-pig pancreatic plasma membrane vesicles was measured at 37°C in 40 mM K-HEPES, pH 7.4, containing 110 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3 and 5 mM ATP. The plasma membrane vesicles were preincubated for 6.5 minutes with the medium. The reaction was started by the addition of an appropriate Ca^{2+} solution containing 130 μM EGTA and varying concentrations of $CaCl_2$ (containing $^{45}CaCl_2$; 200,000 dpm/sample) to give the desired free Ca^{2+} concentrations. Free Ca^{2+} concentrations were determined using a Fortran program as described below. Ca^{2+} uptake was terminated after 5 minutes by filtering an aliquot of the reaction mixture through 0.45 μM Millipore filters (HA 45, Millipore® Co.). The filters were washed with 20 ml of buffer containing 40 mM HEPES, pH 7.4 and 0.25 M sucrose. The washed filters were dried, placed in the liquid scintillation fluor (Unisolve 1®) and counted in a TRI-CARB 4530 scintillation counter (Canberra-Packard Canada Ltd.). The rate of Ca^{2+} transport was determined using the following equation:

$$^{45}Ca^{2+}\text{-uptake} = \frac{(S.C. - B.G.) \times D.F. \times Ca}{(T.C. - B.G.) \times P \times T}$$

where:

S.C. = $^{45}Ca^{2+}$ counts (dpm) in the sample

B.G. = counts (dpm) obtained from scintillant alone

D.F. = correction for incubation volume sampled (=1.21)

Ca = nanomoles of total $CaCl_2$ per tube

T.C. = total radioactivity added to each sample (dpm)

P = mg protein per sample

T = reaction time in minutes

Assay of Ca^{2+}-ATPase Activity

Ca^{2+}-ATPase activity was measured by a modification of the method of Blostein (1970). Unless otherwise stated, plasma membrane vesicles (50 μg/ml) were preincubated for 3 minutes at 37°C in a medium containing 40 mM HEPES, pH 7.4, 130 μM EGTA, 5 mM NaN_3, 0.1 mM ouabain, 500 μM $MgCl_2$ and different amounts of $CaCl_2$ to give the desired free Ca^{2+} concentrations as determined by a Fortran program described below. The reaction was started, in a final reaction volume of 0.2 ml, by the addition of 500 μM ATP (containing γ-^{32}P-ATP; 2 x 10^5 dpm per sample) and terminated, after 30 minutes (except for time-course studies), with ice-cold "TCA stop solution" (consisting of 5% [w/v] trichloroacetic acid, 5 mM Na_2ATP and 2 mM KH_2PO_4). Unhydrolyzed ATP was removed from the reaction mixture by adsorption with a suspension of activated charcoal (0.15 g/ml in 5% [w/v] TCA). Samples were shaken with the charcoal slurry for 5 minutes at room temperature in an Eppendorf Shaker (Brinkman Instruments), then centrifuged at 16,000x*g* for 5 minutes in an Eppendorf microcentrifuge. An aliquot of the supernatant was analyzed for liberated $^{32}P_i$,

by liquid scintillation counting and used to calculate enzymatic ATP hydrolysis. Ca^{2+}-dependent ATPase activity was calculated by subtracting the "basal" activity (in the absence of Ca^{2+}) from the "total" activity (in the presence of Ca^{2+}).

In addition, a modification of the colourimetric assay of Raess & Vincenzi (1980) was used to study the substrate specificity of hydrolytic activity. The reaction was carried out under the same conditions as above, except 1.0 mM ATP or alternative substrates were used in a final reaction volume of 0.4 ml. The reaction was stopped with 0.2 ml of 10% (w/v) sodium dodecyl sulphate (SDS). After the addition of 0.2 ml of 9% (w/v) ascorbic acid, followed by 0.2 ml of ammonium molybdate solution (1.25% [w/v] ammonium molybdate, 6.5% [v/v] H_2SO_4), the absorbance of the samples was read at 660 nm. The phosphate concentration was extrapolated from standard curves performed for each experiment. ATPase activity, expressed in nanomoles per mg protein per minute, was calculated as follows:

For radiometric assay:

$$\text{ATPase Activity} = \frac{(\text{S.C.} - \text{B.G.}) \times \text{ATP} \times \text{D.F.}}{\text{T.C.} \times \text{P} \times \text{T}}$$

where:

S.C. = $^{32}P_i$ counts (cpm) in the sample

B.G. = counts (cpm) obtained from scintillant alone

ATP = nanomoles ATP present in each sample tube

D.F. = dilution factor

$$= \frac{\text{reaction volume} + \text{stop solution} + \text{charcoal volume}}{\text{volume counted}}$$

T.C. = total radioactivity added to each sample (cpm)

P = mg protein per sample

T = reaction time in minutes (=30)

For colourimetric assay:

$$\text{ATPase Activity} = \frac{(\text{S.C.} - \text{B.C.}) \times V}{P \times T}$$

where:

S.C. = phosphate concentration in the sample as obtained from the standard curve (nmoles/ml)

B.C. = phosphate concentration of the blanks

V = reaction volume (0.4 ml)

P = mg protein in the sample

T = reaction time in minutes (=30)

Assay of Na^+/K^+-ATPase Activity

The Na^+/K^+-ATPase assay was similar to the Ca^{2+}-ATPase assay. The assay medium consisted of 40 mM HEPES, pH 7.4, 100 μM EGTA, 500 μM $MgCl_2$, 20 mM KCL, 100 mM NaCl and 500 μM Tris-ATP (containing γ-^{32}P-ATP; 2 x 10^5 dpm per sample). The assay procedure was the same as Ca^{2+}-ATPase. Na^+/K^+-ATPase was taken as the ATPase activity that could be inhibited by 1.5 mM ouabain.

Determination of Endogenous Calmodulin

The concentration of plasma membrane preparations was adjusted to approximately 1 mg/ml protein with 10 mM HEPES, pH 7.4 solution, containing 0.2 mM EDTA. The suspension was incubated for 5 minutes at 95°C and then centrifuged at 40,000x*g* for 30 minutes. The supernatant was collected, and the chelator "neutralized" with $CaCl_2$ (0.2 mM final). The resulting extract was dialyzed against 10 mM NH_4HCO_3, pH 7.0 for 48 hours in the cold room. The

extract was concentrated against crystalline polyethylene glycol. Concentrated samples were quick frozen and stored at -80°C until use. The extracts were assayed for the presence of CaM using erythrocyte plasma membrane Ca^{2+}-ATPase, an enzyme known to be stimulated by CaM (Eibschutz et al., 1984). The CaM-depleted erythrocyte plasma membranes were a generous gift from Dr. Roufogalis's laboratory. These membranes were prepared using the method described by Wang et al. (1988). The assay procedure was similar to the ATPase assay described above.

Phosphorylation of Guinea-pig Acinar Plasma Membranes

Formation of the Ca^{2+}-dependent phosphorylated intermediate of Ca^{2+}-ATPase was studied under conditions similar to those utilized in the ATPase assay described above. The phosphorylation medium consisted of 40 mM HEPES, pH 7.4, 2 mM EDTA or 2 mM $CaCl_2$ and 2 μM ATP (containing γ-^{32}P-ATP; 4 x 10^6 dpm per sample). The membrane preparation and the medium were preincubated separately at 10°C for 10 minutes. The reaction was started by the addition of plasma membranes to the medium. After 15 seconds at 10°C, the reaction was terminated with ice-cold 15% (w/v) TCA. Suspensions were mixed and centrifuged at 1,500x*g* for 10 minutes at 4°C. Pellets were resuspended in 0.5 ml of either 0.6 M hydroxylamine/0.8 M sodium acetate, pH 5.2 or 0.6 M NaCl/0.8 M sodium acetate, pH 5.2 (control). After a 10 minute incubation at room temperature, 1 ml of 15% (w/v) TCA was added to the samples, and the membrane protein was pelleted by centrifugation (10 minutes at 1,500x*g*). The pellet was resuspended in sample buffer and applied to polyacrylamide gels, as described below.

Polyacrylamide Gel Electrophoresis and Autoradiography

Sodium Dodecyl Sulphate Polyacrylamide gels

Polyacrylamide slab gels (5-20%) were cast by the method of Laemmli and Favre (1973). The "separating" gel consisted of a 5-20% gradient of acrylamide-bisacrylamide mixture (30:0.8, w/w), containing 375 mM Tris-Cl buffer, pH 8.8, 0.1% SDS, 1.35-5.75% glycerol, 0.15 mg/ml ammonium persulphate (AP) and 0.03% tetraethyl-methylenediamine (TEMED). The "stacking" gel consisted of 5% acrylamide-bisacrylamide mixture, 315 mM Tris-Cl, pH 6.8, 0.1% SDS, 0.4 mg/ml AP and 0.136% TEMED.

The samples were boiled for 4 minutes in sample buffer (62.5 mM Tris-Cl, pH 6.8 2% [w/v] SDS, 0.5 M 2-mercaptoethanol, 10% [v/v] glycerol and a small amount of Bromophenol Blue) and applied to individual wells. The gels were run at room temperature for approximately 1 hour under constant current of 25 mA, followed by 1.5-2 hours at 50 mA (Biorad Model 1000/500 Power Supply). The running buffer contained 25 mM Tris-Cl, pH 8.3. The protein standards used for estimation of molecular weight (in kilodaltons) were: myosin (200), β-galactosidase (116.25), phosphorylase b (92.5), bovine serum albumin (66.2), ovalbumin (45), bovine carbonic anhydrase (31), soybean trypsin inhibitor (21.5) and lysozyme (14.4). The gels were stained with silver stain to visualize the proteins (see below).

Acid gels

For electrophoresis of phosphorylated samples, the acid-gel system of Lichtner and Wolf (1979) was used. Polyacrylamide gels were prepared as above except Tris-Cl was substituted by 3-(N-Morpholino)propanesulfonic acid (Mops)

and lithium dodecyl sulphate (LiDS) replaced SDS in the gels. The pH of the "separating" gel was 7.0 and that of "stacking" gel was 6.5.

Phosphorylated proteins were precipitated with 15% (w/v) TCA and centrifuged at 1,500x*g*. Pellets were resuspended in 25 µl of sample buffer (50 mM Tris-PO_4, 2.5% LiDS, 0.12 M sucrose, 0.5 M 2-mercaptoethanol and 0.01% (w/v) Bromophenol Blue) and neutralized with 2 M Mops buffer. After incubation at 37°C for 4 minutes, the samples were applied to individual wells and gels were run as above, but at 4°C. The running buffer contained 20 mM Mops, pH 6.5 and 0.2% LiDS. Following electrophoresis, gels were stained with Coomassie Blue to visualize the proteins.

Staining, Destaining and Drying

Silver Staining

When working with small amounts of proteins, the silver staining method of Morrissey (1981) was used due to its high sensitivity. Glass trays were used for staining and gels were handled only with gloves to avoid transferring fingerprints. All solutions were made fresh immediately prior to each step. The gels were fixed in a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 20 minutes. After a 20 minute incubation in 5% (v/v) methanol and 7% (v/v) acetic acid mixture, the gels were transferred to 10% (v/v) glutaraldehyde for 30 minutes. The gels were washed in deionized distilled water for at least 2 hours and then transferred to 5 µg/ml dithiothreitol solution for a 20 minute incubation. After discarding the dithiothreitol, the gels were stained with 0.1% (w/v) silver nitrate for 20 minutes. Excess silver nitrate was removed by rinsing the gels once in deionized distilled water. Superficial silver nitrate was removed by rinsing twice in small volumes of developer (0.0185% [v/v] formaldehyde, 3%

[w/v] $NaCO_3$). The gels were gently shaken in fresh developer until all standards were visible. The developing process was stopped with the rapid addition of 2.3 M citric acid which was prepared before the addition of developer. After a 10 minute incubation, the gels were washed and stored in deionized distilled water until drying.

Coomassie Blue Staining

The gels were stained in a mixture containing methanol, acetic acid, water (5:1:5) and 0.25% (w/v) Coomassie Brilliant Blue R-250 for 30 minutes at room temperature. The gels were then destained in a mixture of methanol, acetic acid and water (5:1:5), for one hour with 3 changes, and then in methanol:acetic acid:water (4:1:15) until the background became clear.

Drying

The gels were dried in a sandwich of clear BioGelWrap sheets (BioDesign Inc., New York) in a Plexiglass frame at room temperature. In some cases, to speed up the drying process, the frame was placed in a fume hood or under a lamp.

Autoradiography

For autoradiography, the gels were dried immediately and exposed to X-ray film (Kodak X-Omat AR) using an intensifying screen (Cronex Lightning Plus, DuPont) for 2-7 days at -80°C. The films were developed using Kodak GBX developer and replenisher and fixed in Kodak GBX fixer and replenisher to visualize the phosphorylated proteins.

Protein Assay

The protein concentrations of membrane preparations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. A full standard curve (0-100 μg protein) was produced for each assay, and the protein concentration was determined by extrapolating from the standard curve.

Determination of Free Calcium Concentration

Free calcium concentrations were determined using the Fortran program "CATIONS" written by Goldstein (1979). Association constants for cations and ligands were obtained from Martell and Smith (1979, 1982) and were corrected for ionic strength, pH and temperature according to the methods described by these authors. For the Ca^{2+} transport assay, the corrected log association constants of chelating ligands (in order of first to fourth proton association) used were: 9.353, 8.733, 2.780 and 2.120 for EGTA; 12.246, 6.184, 3.650 and 2.540 for trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA); 6.664, 4.075, 0.0 and 0.0 for ATP. Association constants for unprotonated and monoprotonated cation-ligand complexes (in order) for the Ca^{2+} transport assay were: 5.414 and 3.802 for Mg-EGTA; 11.225 and 0.0 for Mg-CDTA; 4.188 and 2.194 for Mg-ATP; 10.639 and 5.196 for Ca-EGTA; 13.049 and 0.923 for Ca-CDTA; 3.742 and 1.888 for Ca-ATP. The corrected log association constants of chelating ligands (in order of first to fourth proton association) used for the Ca^{2+}-ATPase assay were: 9.323, 8.703, 2.750 and 2.090 for EGTA; 6.634, 4.045, 0.0 and 0.0 for ATP. Association constants for unprotonated and monoprotonated cation-ligand complexes for the Ca^{2+}-ATPase assay were the same as above.

Statistical Analysis

Student's unpaired t-test was used to compare two means, as required. The null hypothesis was rejected only if the two means were different at the 0.05 significance level.

RESULTS

THE PLASMA MEMBRANE PREPARATION

The viability of isolated acinar cells, as determined by trypan blue exclusion studies, was consistently 90% or higher. There was minimal bacterial contamination. Figure 5 shows the recovery data for protein, Ca^{2+}-ATPase and Na^+/K^+-ATPase activities in various fractions following homogenization and differential centrifugation. Almost 30% protein was lost in the first pellet, P_1 (the 180x*g* pellet). This pellet also accounted for more than 50% of the Na^+/K^+-ATPase activity and 27% of the Ca^{2+}-ATPase activity. In later experiments, the pellet P_1 was re-homogenized and centrifuged to recover some of this lost activity. The supernatants were pooled for the next step. Very little activity was lost in the second pellet, P_2 (the 1,000x*g* pellet). The recovery of protein, Ca^{2+}-ATPase activity and Na^+/K^+-ATPase activity in the P_3 fraction (the 150,000x*g* fraction) was 19%, 23% and 35%, respectively. After sucrose density gradient centrifugation, a large fraction of protein (7%) and the two ATPase activities (9% Ca^{2+}-ATPase activity and 5% Na^+/K^+-ATPase activity) was lost in the final pellet, P_4. However, significant amounts of protein (1.3%), Ca^{2+}-ATPase activity (2.6%) and Na^+/K^+-ATPase activity (6.5%), were recovered in the 27/35% sucrose interface. The other two sucrose interfaces contained very little protein or marker activities.

Compared to the homogenate, P_3 (the fraction used for the source of plasma membranes) showed a 2-fold enrichment of ouabain-sensitive Na^+/K^+-ATPase activity, the basolateral plasma membrane marker, while $(Ca^{2+}+Mg^{2+})$-ATPase activity was not enriched (Table II). However, material collected from the 27/35% interface following sucrose density gradient centrifugation was found to be enriched 17-fold in Na^+/K^+-ATPase and 3.4-fold in $(Ca^{2+}+Mg^{2+})$-

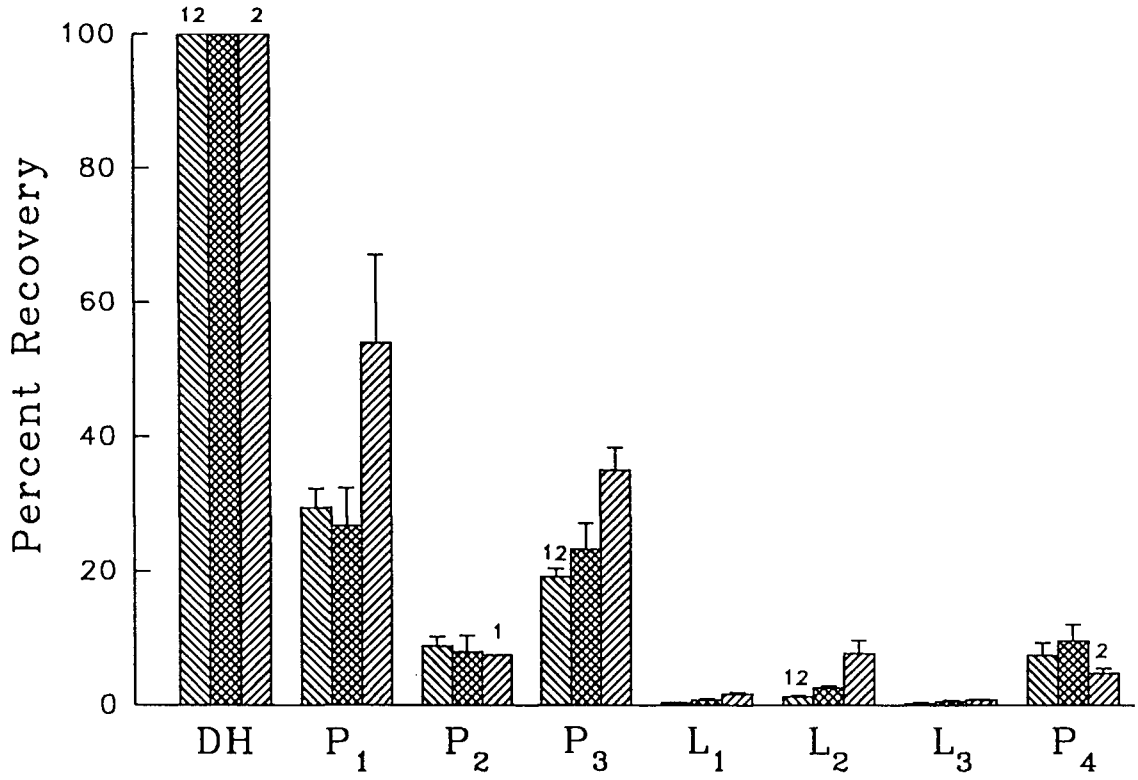

Figure 5. Recovery of protein, Ca^{2+}-ATPase activity and Na^{+}/K^{+}-ATPase activity in different centrifugation fractions of guinea-pig pancreatic acinar plasma membrane preparations. The results represent the mean + S.E.M. for three experiments, unless otherwise indicated by a number above the bar. DH - dilute homogenate, P_1 - 180x*g* pellet, P_2 - 1,000x*g* pellet, P_3 - 150,000x*g* pellet, L_1, L_2, and L_3 - the 10/27%, 27/35% and 35/38% interfaces from the sucrose density gradient, P_4 - pelleted fraction from the sucrose density gradient. ▨ - Protein, ▩ - Ca^{2+}-ATPase, ▨ - Na^{+}/K^{+}-ATPase.

Table II. A comparison of the specific activities (SA) at different stages of the guinea-pig pancreatic acinar plasma membrane preparation. The results represent means of three experiments except where indicated within parentheses.

Fraction	$(Ca^{2+}+Mg^{2+})$-ATPase		Na^+/K^+-ATPase	
	SA	Enrichment	SA	Enrichment
Dilute Homogenate	9.58	1.0	3.40	1.0
150,000x*g* Fraction (P_3)	9.53	1.0	7.13	2.1
Plasma Membranes (27/35% interface)	32.33	3.4	58.68	17.2

The activities were measured as described in the **METHODS** and are expressed in nanomoles ATP hydrolyzed/mg/min. The Mg^{2+} concentration was 0.5 mM and the free Ca^{2+} was 1.0 μM in the assay for $(Ca^{2+}+Mg^{2+})$-ATPase activity. Na^+/K^+-ATPase was taken as the ATPase activity that could be inhibited by 1.5 mM ouabain.

ATPase activity (Table II). This fraction was designated the plasma membrane-enriched fraction. Sodium azide (5 mM) was included in all reaction media to inhibit any residual mitochondrial ATPase which may have been present in this fraction.

OPTIMIZATION OF ASSAY CONDITIONS

To determine optimal conditions for assay of ATPase and Ca^{2+}-transport activities, the effects of varying membrane protein concentration within reaction media were investigated. The $^{45}Ca^{2+}$-uptake studies showed a near linear increase with protein concentrations up to 180 μg/ml (Fig. 6). A linear increase was also observed in the ATPase activity with protein concentrations up to 190 μg/ml (Fig. 7). Subsequent studies were, therefore, carried out at membrane protein concentrations of 15-50 μg/ml for the radiometric ATPase assay and 100-125 μg/ml for the colourimetric ATPase assay, while approximately 120 μg/ml protein was used in the Ca^{2+}-transport experiments.

In other experiments, the time-course for the ATPase activity was studied. The Ca^{2+}-dependent ATP hydrolysis was found to be approximately linear for at least 30 minutes (Fig. 8). A 30 minute reaction time was selected for all subsequent ATPase determinations.

In our experiments, guinea-pig pancreatic acinar plasma membrane preparations typically showed maximal Ca^{2+}-dependent ATPase activities ranging between 5 and 15 nmoles/mg/min. Freeze-thaw experiments were conducted in an attempt to expose possible "latent" ATPase sites within the plasma membranes. Figure 9 shows the effect of freeze-thawing on Ca^{2+}-dependent, Mg^{2+}-dependent and Total ATPase activities. Freeze-thawing the plasma membranes up to 5 times failed to produce a measurable increase in any of the three ATPase activities (Fig. 9). This suggested that the enzymatic sites

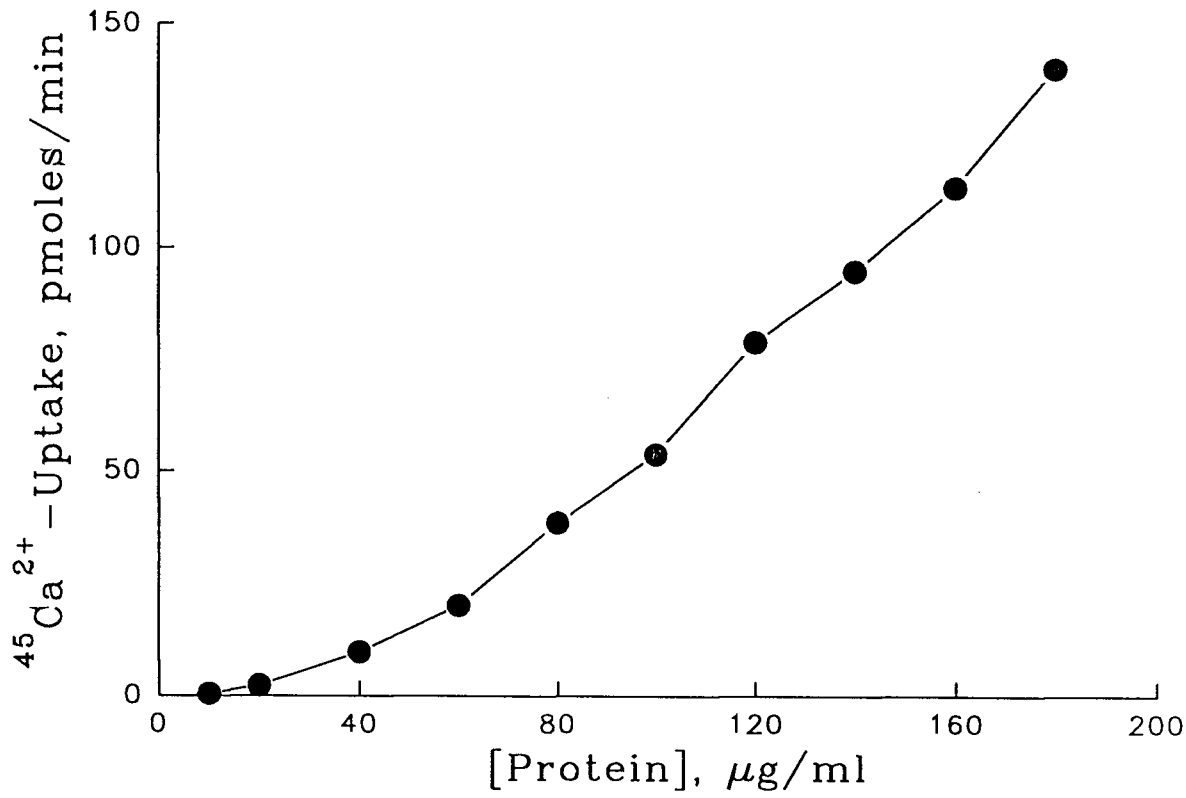

Figure 6. Effect of varying protein concentration on $^{45}Ca^{2+}$-uptake in guinea-pig pancreatic acinar plasma membranes. The experiments were done in the presence of 1.0 μM free Ca^{2+} and 5 mM added Mg^{2+}. Other reaction conditions are described in the **METHODS**. The results represent the mean of two separate experiments.

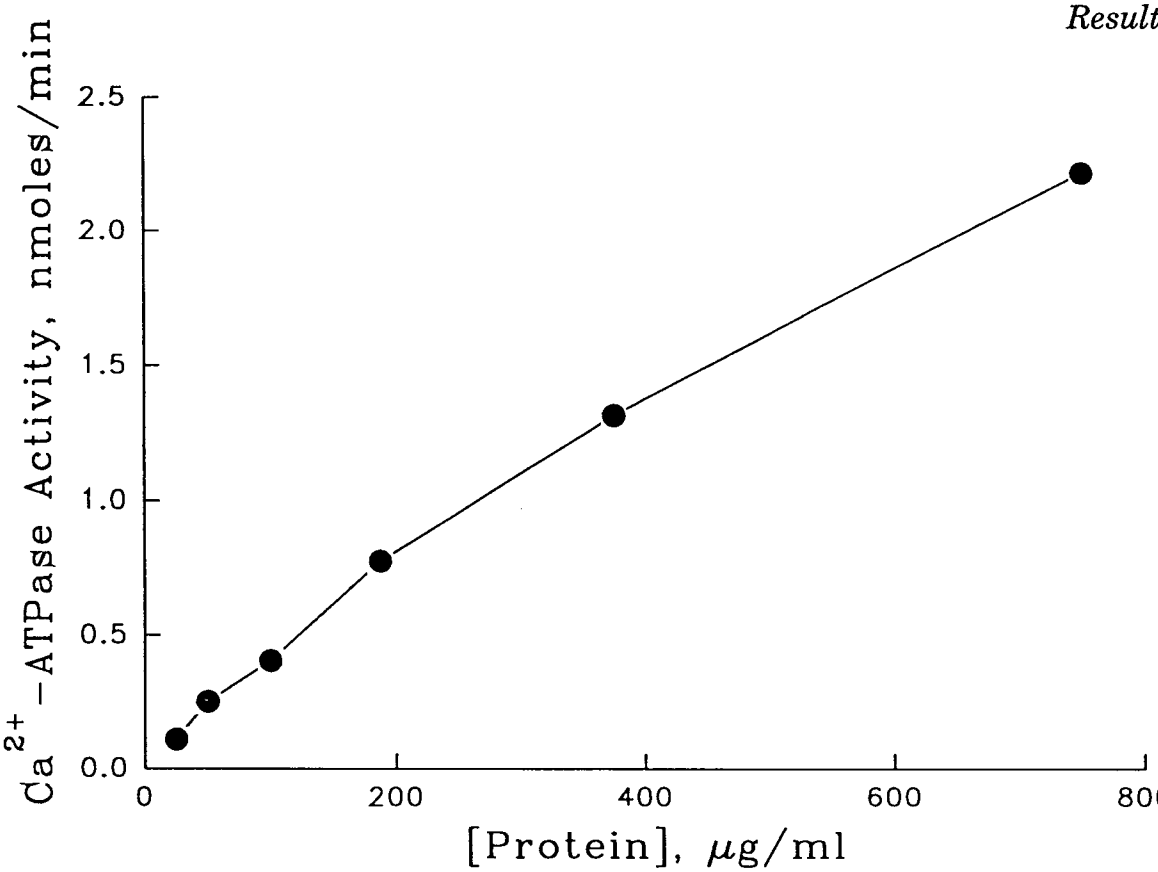

Figure 7. Effect of varying protein concentration on Ca^{2+}-dependent ATPase activity in guinea-pig pancreatic acinar plasma membranes. The experiments were carried out in the presence of 1.0 μM free Ca^{2+} and in the absence of added Mg^{2+}. Other reaction conditions are described in the **METHODS**. The results represent the mean of two separate experiments.

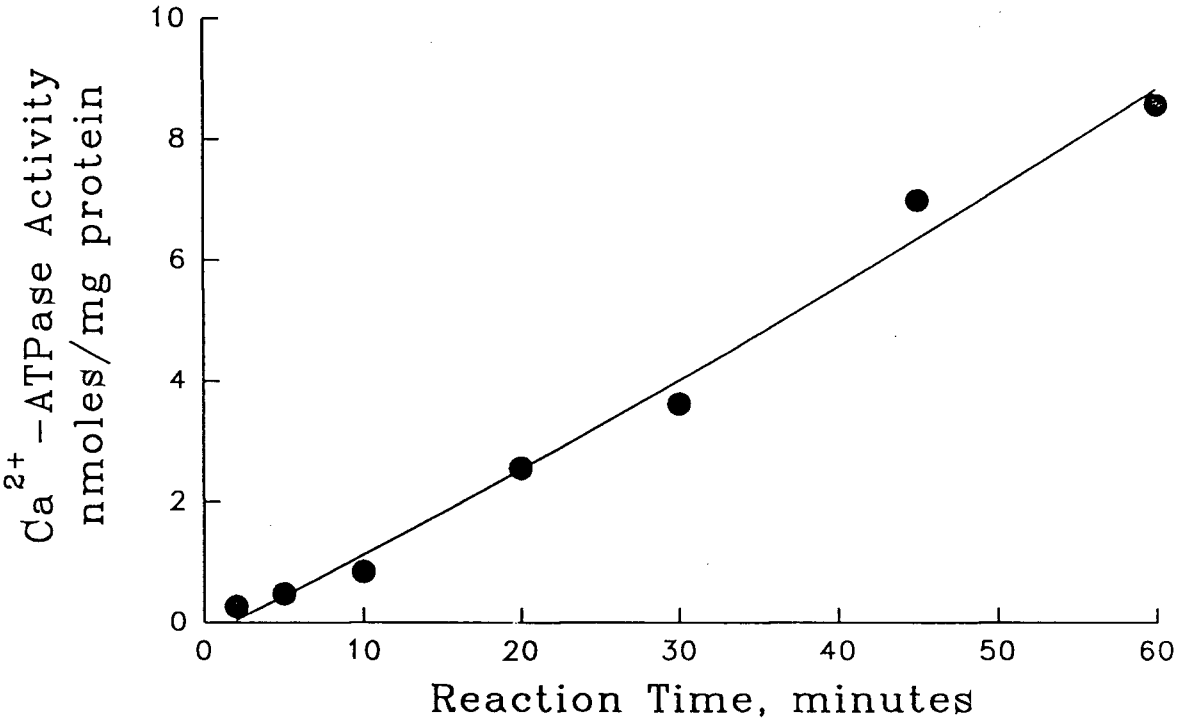

Figure 8. Time-course of Ca^{2+}-ATPase activity. The experiments were done in the presence of 1.0 μM free Ca^{2+} and zero added Mg^{2+}. The results represent the means of two to three separate experiments.

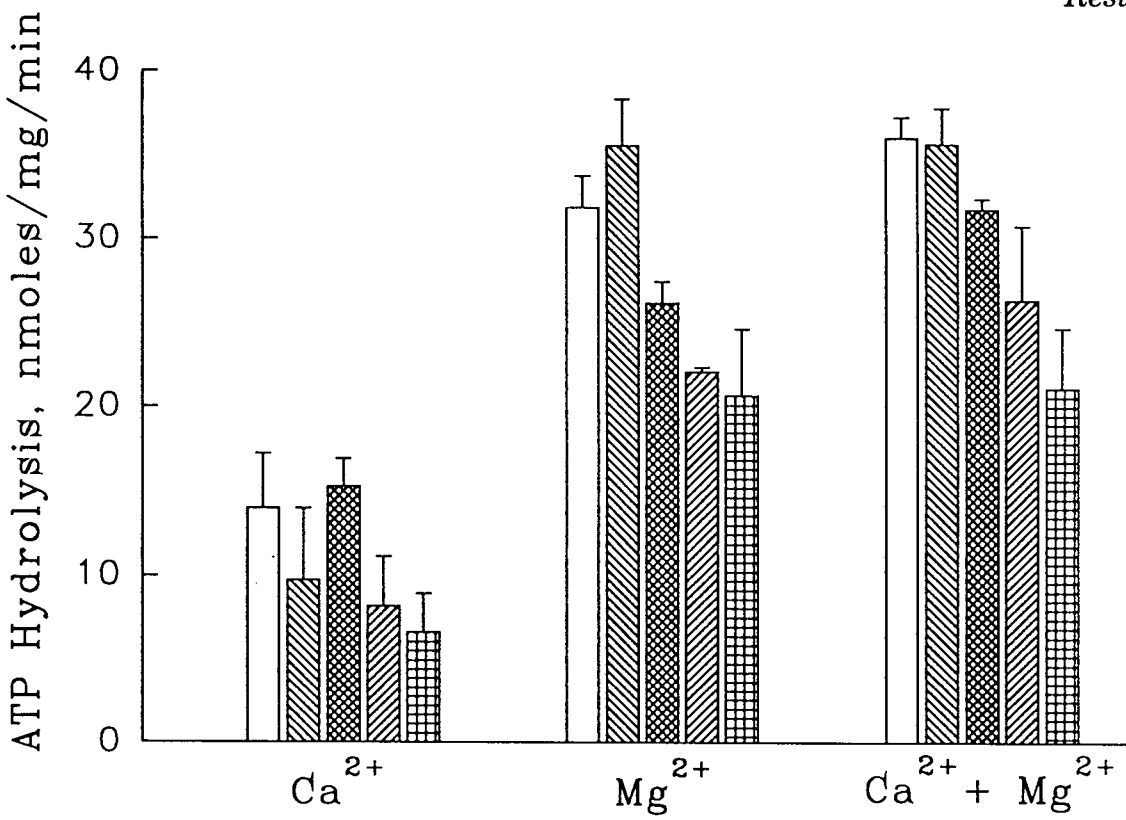

Figure 9. Effect of freeze-thawing on Ca^{2+}-dependent, Mg^{2+}-dependent and total ATPase activity. The results represent the mean ± S.E.M. for three experiments. ▭ - 0 freeze-thaw, ▨ - 1 freeze-thaw, ▩ - 2 freeze-thaw, ▨ - 3 freeze-thaw, ▦ - 4 freeze-thaw.

were already maximally exposed to the medium prior to freeze-thawing. Freeze-thawing the plasma membranes 2 or 4 times actually tended to cause inhibition of Mg^{2+}-dependent and total ATPase activities. The Mg^{2+}-dependent activity was also significantly inhibited after 3 freeze-thawings (Fig. 9).

CHARACTERIZATION OF Ca^{2+} TRANSPORT

Effects of Ca^{2+} and Calmodulin

The ATP-dependent transport of $^{45}Ca^{2+}$ by guinea-pig pancreatic acinar plasma membrane vesicles was stimulated by Ca^{2+} in a concentration-dependent manner (Fig. 10). Maximal uptake was achieved at approximately 1.0 μM free Ca^{2+} and remained at this level up to 6 μM Ca^{2+}. Double-reciprocal analysis of these data produced the following kinetic parameters: K_{Ca} = 0.04 ± 0.01 μM (n=5) and V_{max} = 0.83 ± 0.09 nmoles/mg/min (n=5). Calmodulin (6 μg/ml) produced no significant effect on K_{Ca} (0.05 ± 0.01 μM, n=5) nor on V_{max} (0.86 ± 0.14 nmoles/mg/min, n=5).

Effect of Mg^{2+}

Addition of Mg^{2+} to the reaction medium produced a concentration-dependent stimulation of ATP-dependent Ca^{2+}-transport (Fig. 11). The rate of Ca^{2+}-transport reached a maximum at approximately 2 mM total Mg^{2+}. The half-maximal rate of Ca^{2+}-uptake was produced at 0.3 ± 0.04 mM total Mg^{2+} (4.3 ± 0.5 μM free Mg^{2+}). Approximately 25% of the maximal Ca^{2+}-uptake activity was expressed in the absence of added Mg^{2+} (Fig. 11). This level of Ca^{2+} transport activity may have resulted from the presence of endogenous Mg^{2+}. Therefore, experiments using CDTA, a chelator which is relatively more specific for Mg^{2+} than EGTA or EDTA, were carried out to examine this possibility. In

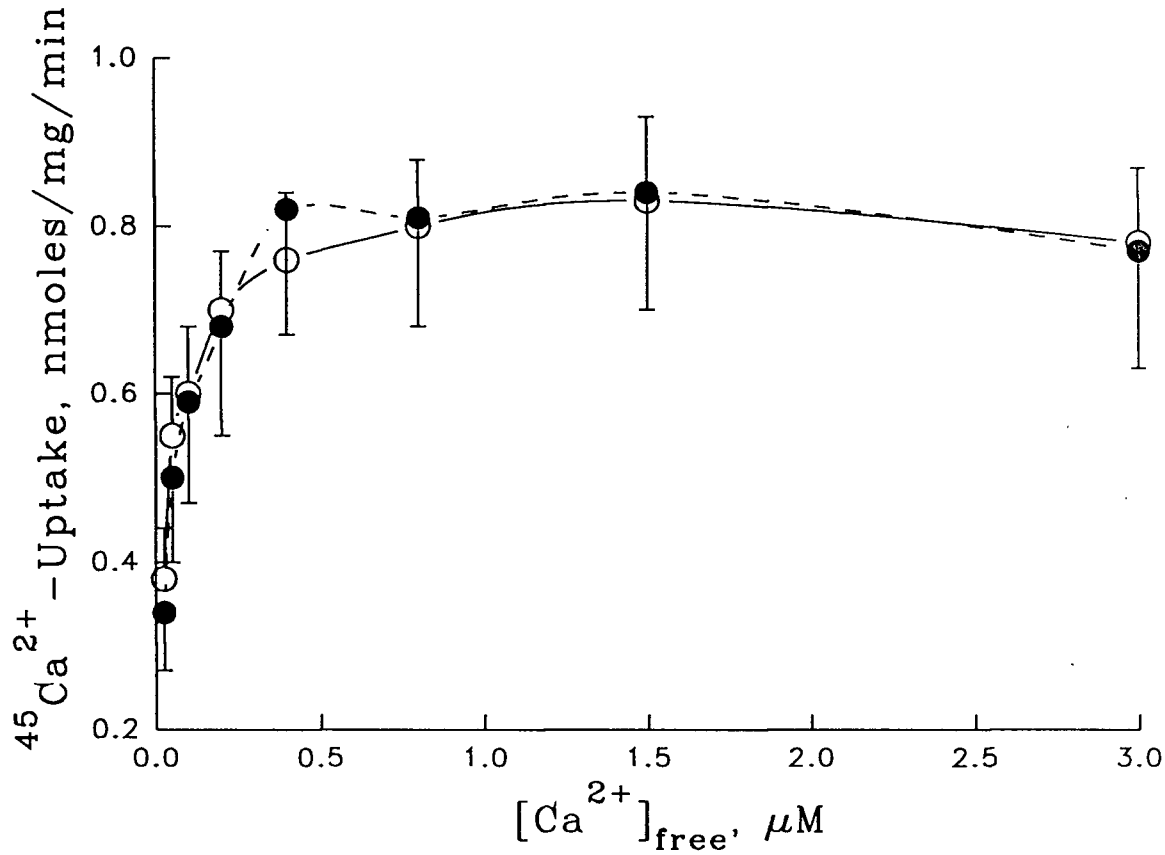

Figure 10. Ca^{2+} activation of $^{45}Ca^{2+}$ uptake by guinea-pig pancreatic acinar plasma membrane vesicles. ATP-dependent calcium transport in the absence (○) and presence (●) of 6 μg/ml calmodulin was measured as described in the **METHODS**. The experiments were carried out in the presence of 5 mM added Mg^{2+}. The results represent the mean ± S.E.M. for five experiments.

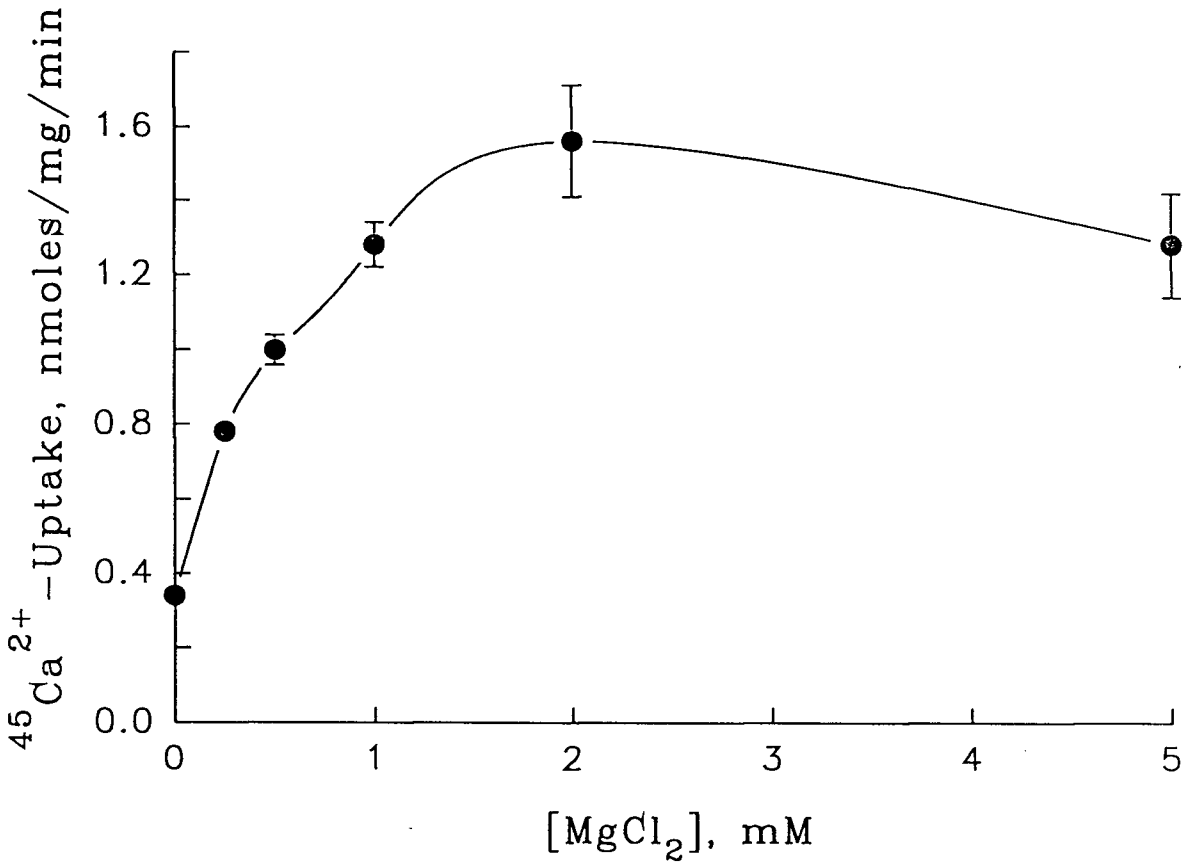

Figure 11. Effect of Mg^{2+} on $^{45}Ca^{2+}$-uptake in guinea-pig pancreatic acinar plasma membrane vesicles. Uptake of $^{45}Ca^{2+}$ was measured as described in the **METHODS**. The free Ca^{2+} concentration was maintained at 1.5 μM. The results represent the mean ± S.E.M. for three experiments.

the absence of added Mg^{2+}, CDTA produced substantial inhibition of $^{45}Ca^{2+}$ transport (Fig. 12). The inhibition appeared to be biphasic: up to 30% of $^{45}Ca^{2+}$-uptake was inhibited by as little as 0.1 mM CDTA, while roughly 60% could be inhibited by 0.8 mM CDTA. It was not feasible to use CDTA concentrations higher than 0.8 mM and still accurately determine free Ca^{2+} concentrations.

Substrate Specificity

Figure 13 shows the rate of $^{45}Ca^{2+}$ transport by guinea-pig pancreatic acinar plasma membrane vesicles in the presence of different nucleotide substrates. The transport process appeared to be highly specific for ATP. Other substrates supported very little Ca^{2+} transport, the highest being 16% of maximum produced by cytosine 5'-triphosphate (CTP). As expected, *p*-nitrophenyl phosphate (pNPP) was unable to support any Ca^{2+} transport.

CHARACTERIZATION OF Ca^{2+}-ATPASE ACTIVITY

To make a direct comparison, ATPase activity was studied in the same membrane fraction as used to measure Ca^{2+} transport. As apparent from the results below, the ATPase activity showed somewhat different properties than the Ca^{2+} transport activity.

Effect of Mg^{2+}

In our studies, Mg^{2+} produced a concentration-dependent stimulation of "Basal" ATP hydrolysis (i.e. the activity measured in the absence of added Ca^{2+}) (Fig. 14). The activity reached a maximum at approximately 500 μM $MgCl_2$. Figure 15 shows the effects of varying Mg^{2+} concentration on the Ca^{2+} activation of Ca^{2+}-dependent ATPase activity. Ca^{2+} was able to stimulate the latter activity in the complete absence of added Mg^{2+}. Increasing Mg^{2+}

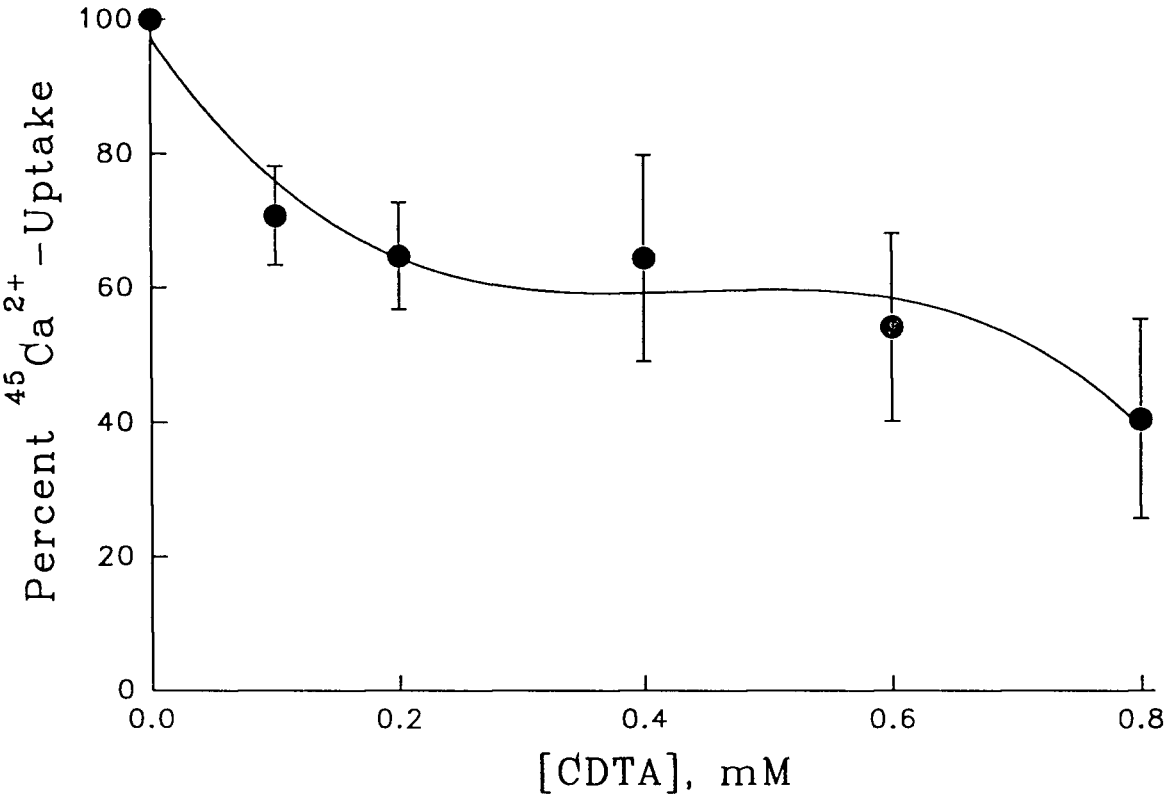

Figure 12. Effect of CDTA on $^{45}Ca^{2+}$ uptake in guinea-pig pancreatic acinar plasma membrane vesicles. The transport of Ca^{2+} was measured in the presence of 1.5 μM free Ca^{2+} and in the absence of added Mg^{2+}. One hundred percent activity corresponded to 0.432 nmoles/mg/min. The results represent the mean ± S.E.M. for five experiments.

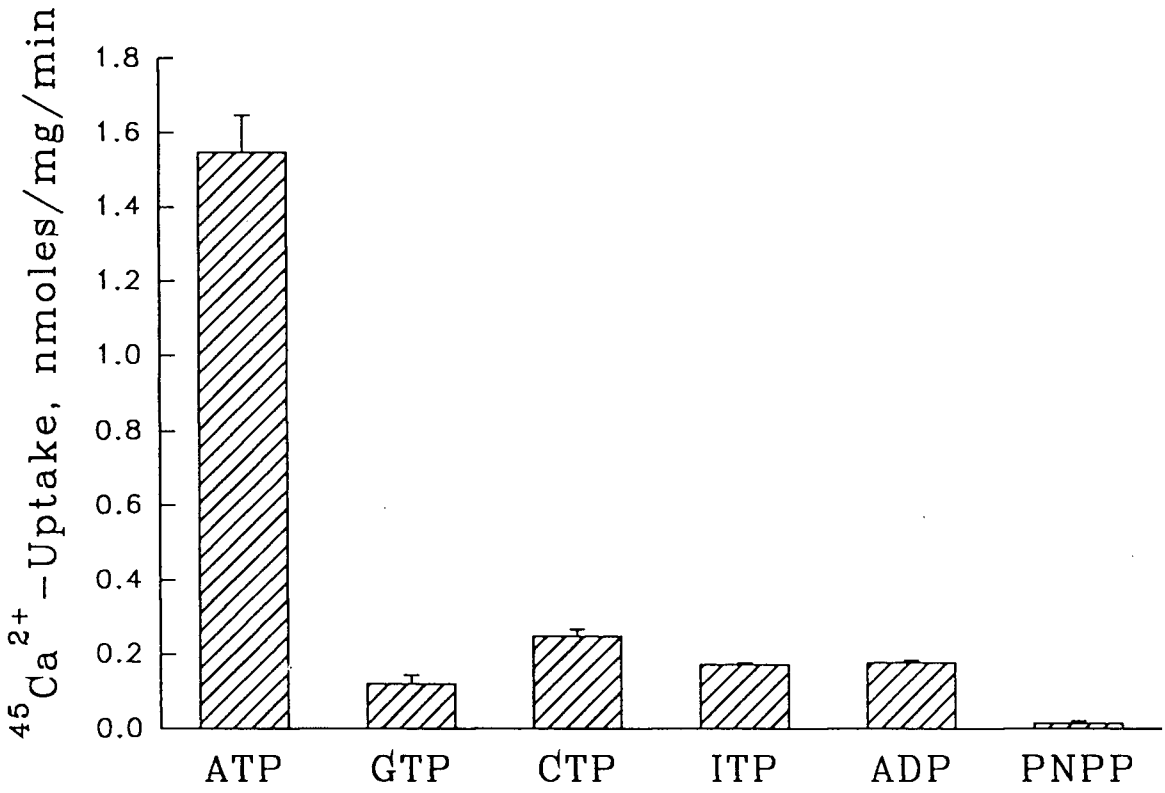

Figure 13 $^{45}Ca^{2+}$-uptake in the presence of different nucleotide substrates in guinea-pig pancreatic acinar plasma membrane vesicles. Ca^{2+}-uptake was measured at 1.5 μM free Ca^{2+} and in the presence of the substrates indicated. The results represent the mean ± S.E.M. for three experiments.

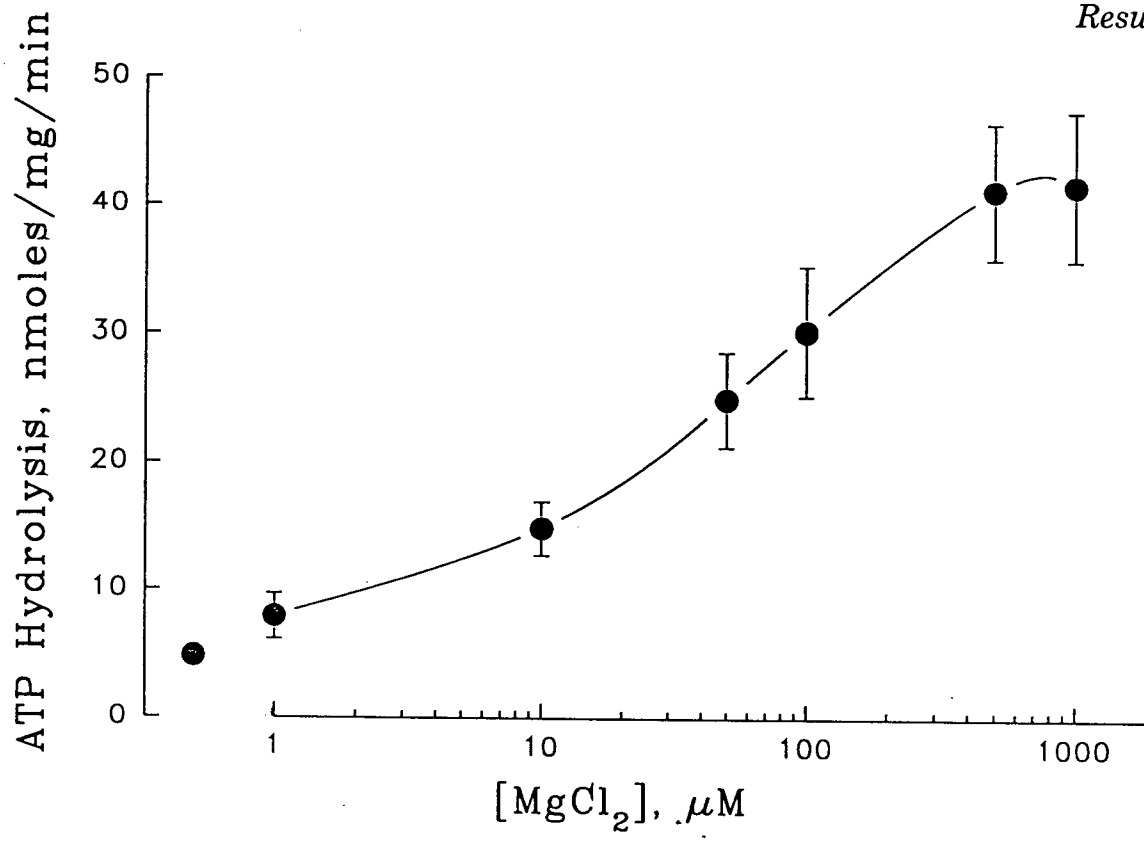

Figure 14. Effect of Mg^{2+} on Basal ATPase activity. There was no added Ca^{2+} present. The results represent the mean ± S.E.M. for three experiments.

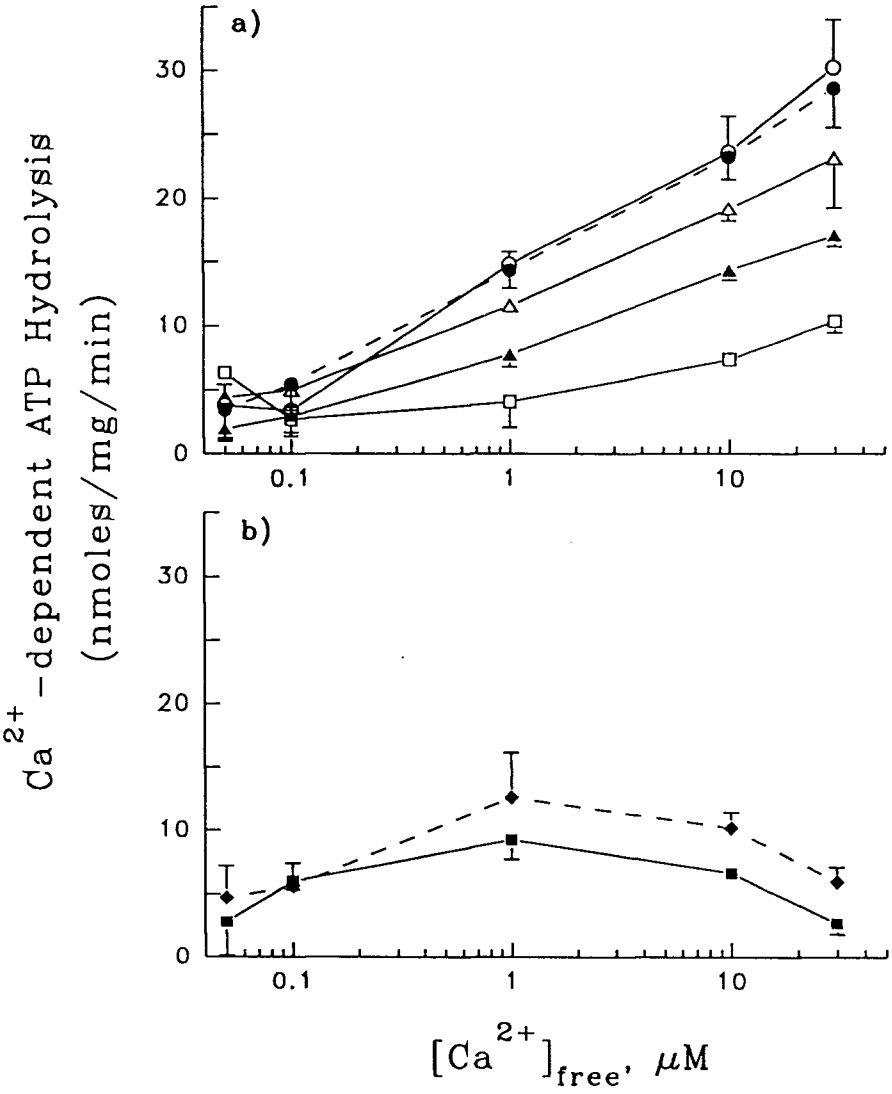

Figure 15. Effect of calcium on Ca^{2+}-dependent ATPase activity in the absence and presence of various magnesium concentrations. **a)** The ATPase activity was assayed in the absence (○), or presence of 1.0 μM (●), 10 μM (Δ), 50 μM (▲) or 100 μM (□) $MgCl_2$. **b)** The ATPase activity was assayed in the presence of 500 μM (■) or 1.0 mM (◆) $MgCl_2$. The Ca^{2+}-dependent activity was calculated by subtracting the basal activity (measured in the absence of Ca^{2+} and the presence of indicated Mg^{2+}) from the total activity (measured in the presence of both Ca^{2+} and Mg^{2+}). The results represent the mean ± S.E.M. for three experiments.

concentrations appeared to inhibit Ca^{2+}-dependent ATP hydrolysis in a concentration-dependent manner (Fig. 15a). However, at high Mg^{2+} concentrations (0.5 and 1.0 mM), this inhibition was lost and Ca^{2+}-dependent ATPase activity increased, peaking at 1 μM free Ca^{2+}. At Ca^{2+} concentrations higher than 1 μM, the Ca^{2+}-dependent activity declined (Fig. 15b). These data indicate the possible presence of two different Ca^{2+}-dependent ATP hydrolytic activities - a low affinity Ca^{2+}(or Mg^{2+})-stimulated ATPase and a high affinity Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase.

Effect of Ca^{2+}, Calmodulin and K^+

Further investigation of the high affinity Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase (in the presence of 500 μM $MgCl_2$) showed that increasing Ca^{2+} concentrations stimulated ATP hydrolysis in a concentration-dependent manner (Fig. 16). Kinetic analysis indicated a V_{max} of 6.04 ± 0.78 nmoles/mg/min and a K_{Ca} of 0.076 ± 0.022 μM. Figure 16 also shows the effect of 6 μg/ml CaM on Ca^{2+}-ATPase activity. As apparent from the graphs, CaM did not significantly stimulate this activity. The calculated V_{max} was 7.13 ± 0.99 nmoles/mg/min, while the K_{Ca} was 0.072 ± 0.018 μM. In other experiments, the effect of potassium on Ca^{2+}-ATPase was studied. Various potassium concentrations tested (1-300 mM) were unable to affect this Ca^{2+}-dependent ATP hydrolysis (Fig. 17 shows partial data).

Formation of the Phosphorylated Intermediate of the Calcium Pump

Autoradiograms of phosphorylation experiments using plasma membranes from guinea-pig pancreatic acini showed the formation of a Ca^{2+}-dependent phosphoprotein with an apparent molecular weight of approximately 100,000 daltons (Fig. 18B, Lane 1). This phosphoprotein could be completely

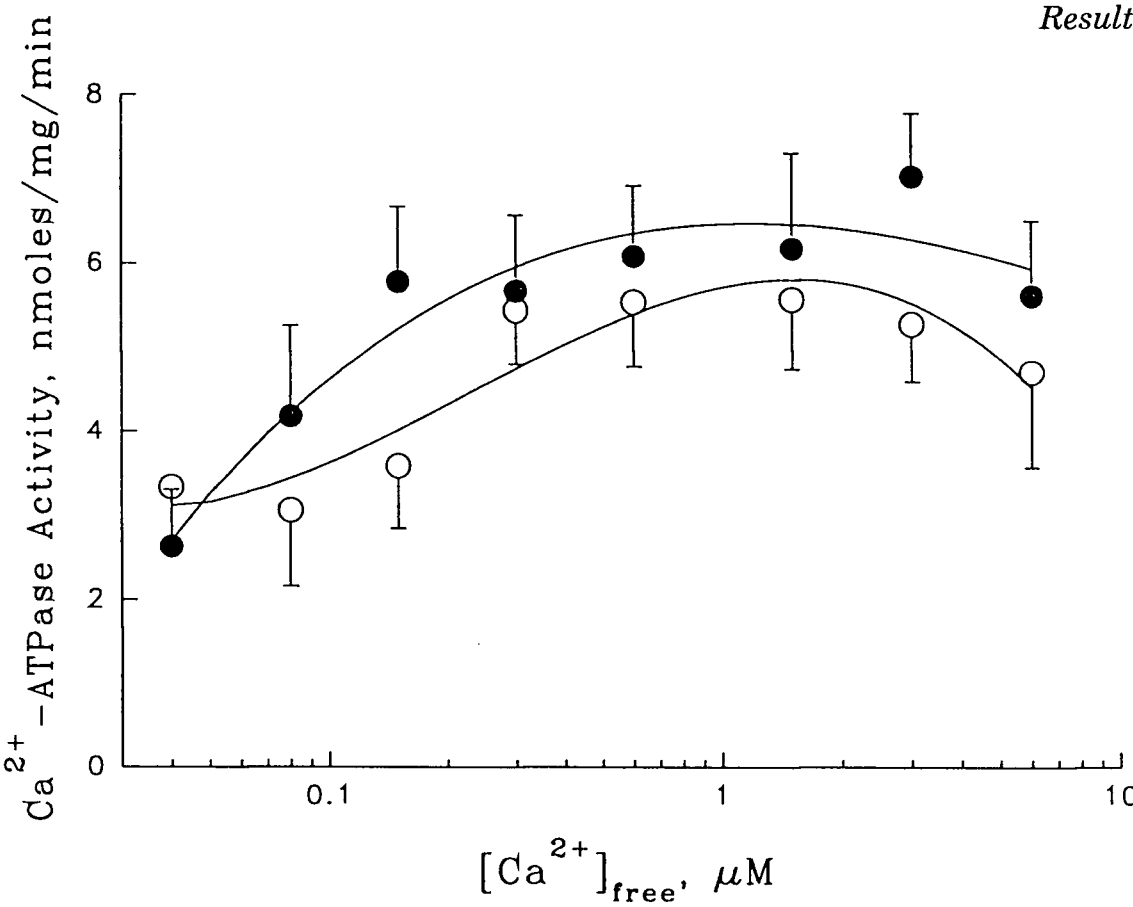

Figure 16. Effect of Ca^{2+} on Ca^{2+}-dependent ATPase activity in the absence (○) or presence (●) of 6 μg/ml calmodulin. The experiments were carried out as described in the **METHODS**. The results represent the mean ± S.E.M. for five experiments.

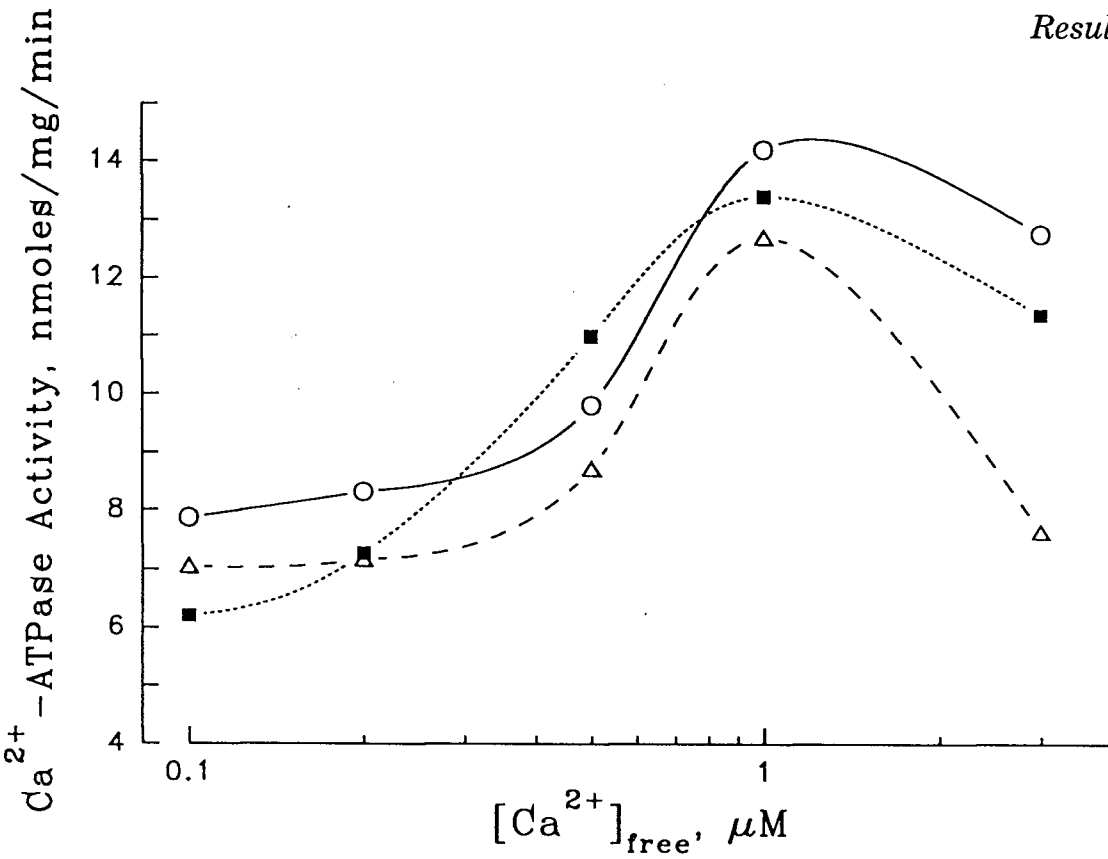

Figure 17. Effect of calcium on Ca^{2+}-dependent ATPase activity in the absence and presence of various potassium concentrations. The potassium concentrations used were: ○ - 0, ■ - 100 mM, Δ - 300 mM. The results represent the average of two experiments.

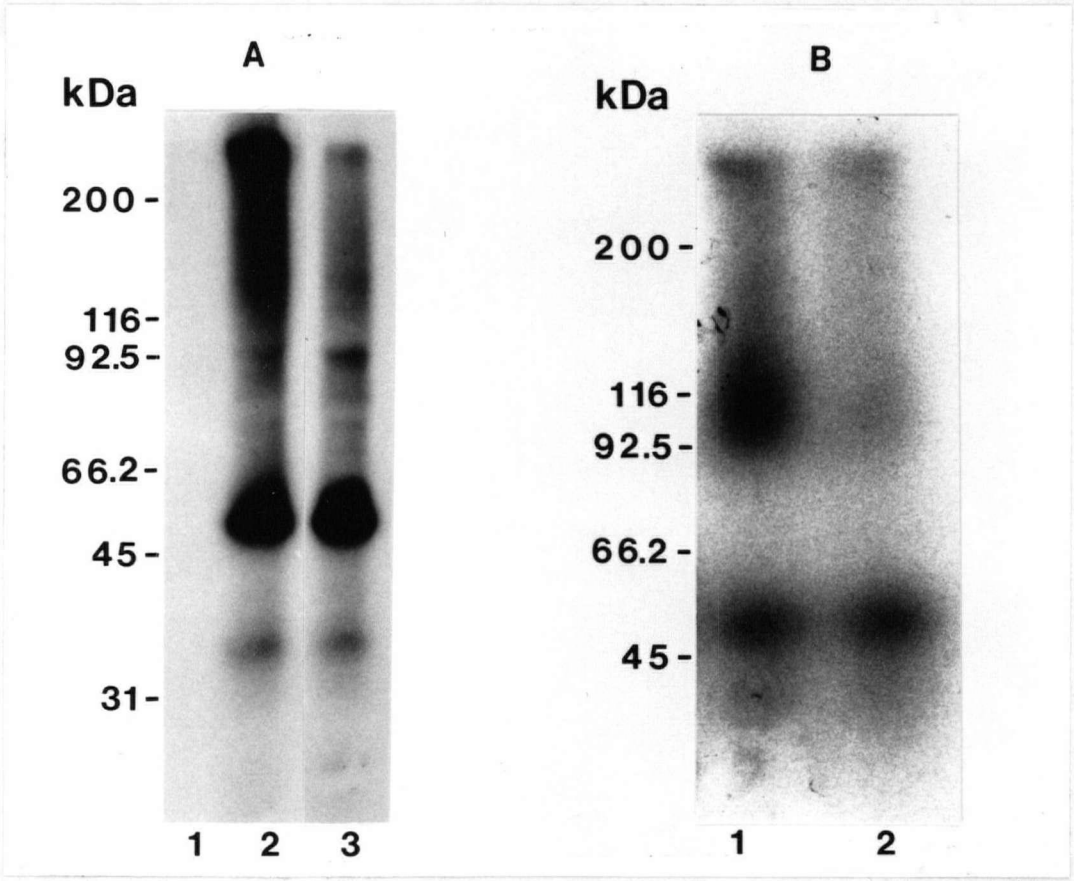

Figure 18. Autoradiogram of phosphorylated proteins in guinea-pig pancreatic acinar plasma membranes. The phosphorylation was carried out in the presence of 2 mM Ca^{2+}. **A** - Mg^{2+}-dependent phosphorylation; *Lane 1* - No Mg^{2+} added, *Lane 2* - 0.5 mM $MgCl_2$, *Lane 3* - 0.5 mM $MgCl_2$ + 0.6 M hydroxylamine. **B** - Ca^{2+}-dependent phosphorylation in the presence of 0.5 mM $MgCl_2$; *Lane 1* - No hydroxylamine, *Lane 2* - 0.6 M hydroxylamine. Molecular weight markers (in kilodaltons) are shown on the left.

hydrolyzed by 0.6 M hydroxylamine (Fig. 18B, Lane 2). The presence of a second phosphoprotein (M_r approx. 50,000) was also detected. However, the phosphorylation of this latter protein appeared to be Mg^{2+}-dependent and was not sensitive to hydroxylamine treatment (Fig. 18A,) suggesting the formation of an ester phosphate bond, instead of a typical ATPase acyl phosphate bond. No phosphorylation was observed in the absence of Ca^{2+} and Mg^{2+}.

Substrate Specificity

A colourimetric assay was used in a systematic study of the hydrolysis of different nucleotide phosphate substrates by Guinea-pig pancreatic acinar plasma membrane preparations. The results showed that in addition to ATP, membranes were able to hydrolyze GTP, CTP, ITP and ADP (Fig. 19). Unlike Ca^{2+}-transport, the nucleotide triphosphatase activity showed little specificity for ATP. GTP appeared to be the favoured substrate; the Ca^{2+}-dependent hydrolysis of GTP, ITP and CTP (measured in the presence of 5 mM Mg^{2+}) was 172%, 74% and 48%, respectively, compared with ATP (Fig. 19a). The hydrolysis of ADP was 20% compared to ATP, and the enzyme showed 18% Ca^{2+}-dependent cleavage of pNPP. Although ATP appeared to be the most favoured substrate for Ca^{2+}-dependent hydrolysis in the absence of Mg^{2+}, the enzyme did not show complete substrate specificity; hydrolysis of ITP, CTP and GTP was 73%, 66% and 56%, respectively, compared to ATP (data not shown).

Mg^{2+}-dependent hydrolysis of GTP also exceeded that of other substrates used (Fig. 19b). ATP appeared to be the least favourable triphosphate substrate for the Mg^{2+}-dependent activity while ADP or pNPP were poorly hydrolysed.

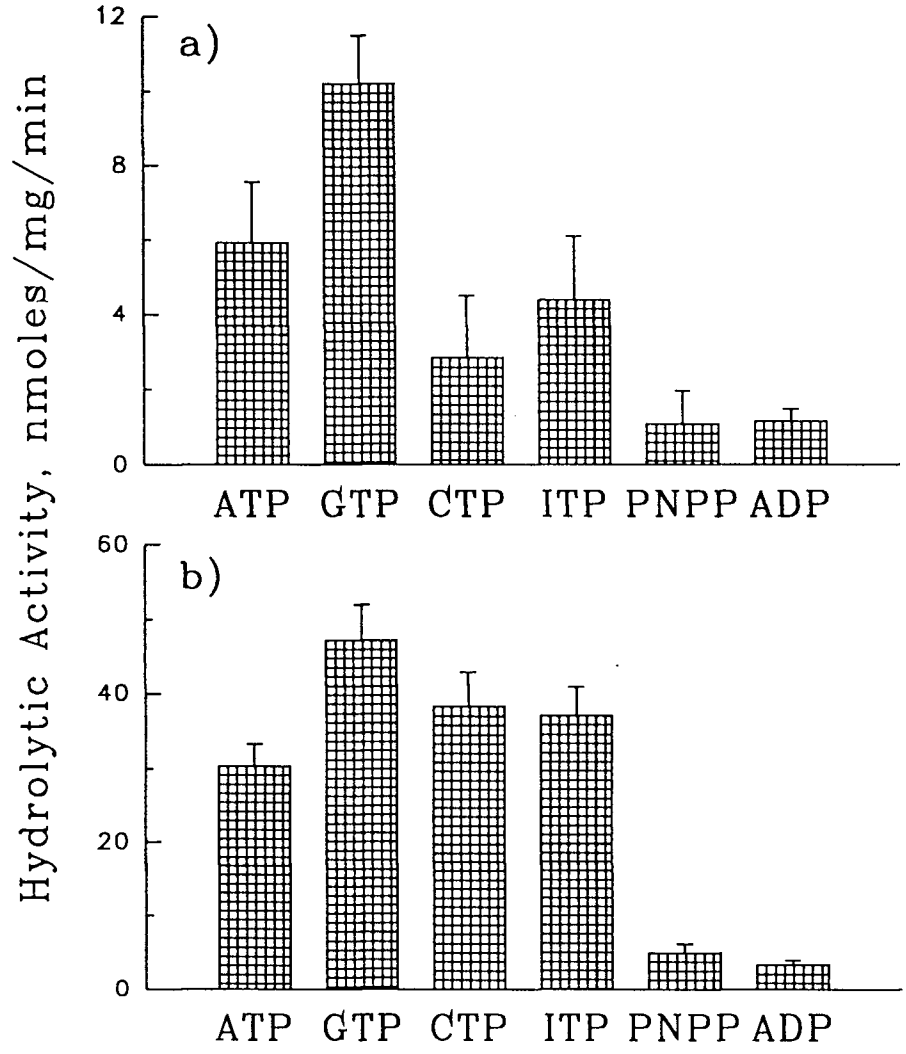

Figure 19. Substrate specificity of Ca^{2+}-dependent and Mg^{2+}-dependent hydrolytic activities in guinea-pig pancreatic acinar plasma membranes. Hydrolytic activity was determined spectrophotometrically as described in the **METHODS**. **a)** Ca^{2+}-dependent hydrolysis was determined in the presence of 5 mM Mg^{2+} and by subtracting the basal activity (i.e. activity measured in the absence of Mg^{2+}) from the total activity measured in the presence of 1 μM free Ca^{2+}. **b)** The Mg^{2+}-dependent hydrolysis was determined as above, but in the presence of 5 mM Mg^{2+} and zero Ca^{2+}. The results represent the mean ± S.E.M. for four experiments.

REGULATION OF Ca^{2+}-TRANSPORT AND Ca^{2+}-ATPase BY PROTEIN KINASES AND INOSITOL PHOSPHATES

Regulation by Protein Kinase A

As shown in Figure 20 and Table III, 400 units/ml catalytic subunit of protein kinase A (C-subunit) stimulated Ca^{2+}-transport into plasma membrane vesicles more than two-fold (V_{max} = 1.26 ± 0.29 nmoles/mg/min for controls; 2.50 ± 0.33 nmoles/mg/min for C-subunit stimulated, $P < 0.05$). The affinity for Ca^{2+} was unaffected (0.18 ± 0.07 μM Vs. 0.17 ± 0.1 μM). In other experiments, 300 units/ml C-subunit produced no statistically significant stimulation of Ca^{2+}-dependent ATPase activity except at 0.08 μM Ca^{2+} (Fig. 21). The V_{max} (control = 4.35 ± 0.94 and C-subunit = 5.18 ± 0.82) and K_{Ca} (control = 0.20 ± 0.15 μM and C-subunit = 0.05 ± 0.01 μM) were also unaffected.

To determine whether stimulation by an endogenous protein kinase A contributed to the Ca^{2+}-transport or Ca^{2+}-ATPase activity observed, the effects of a specific protein kinase A inhibitor, HA 1004, were studied. Under our experimental conditions HA 1004 failed to inhibit either Ca^{2+}-transport (Table III) or Ca^{2+}-ATPase (Fig. 21) activities. The inhibitor also failed to affect the stimulatory effect of exogenously added C-subunit on Ca^{2+}-uptake (Table III).

Regulation by CaM

As reported above, exogenous CaM had no effect on either Ca^{2+}-transport (Fig. 10) or Ca^{2+}-dependent ATPase activity (Fig. 16) in guinea-pig pancreatic acinar plasma membranes. To further understand the regulation of these activities, effects of three putative CaM inhibitors, trifluoperazine (TFP), compound 48/80 and CGS 9343B were investigated.

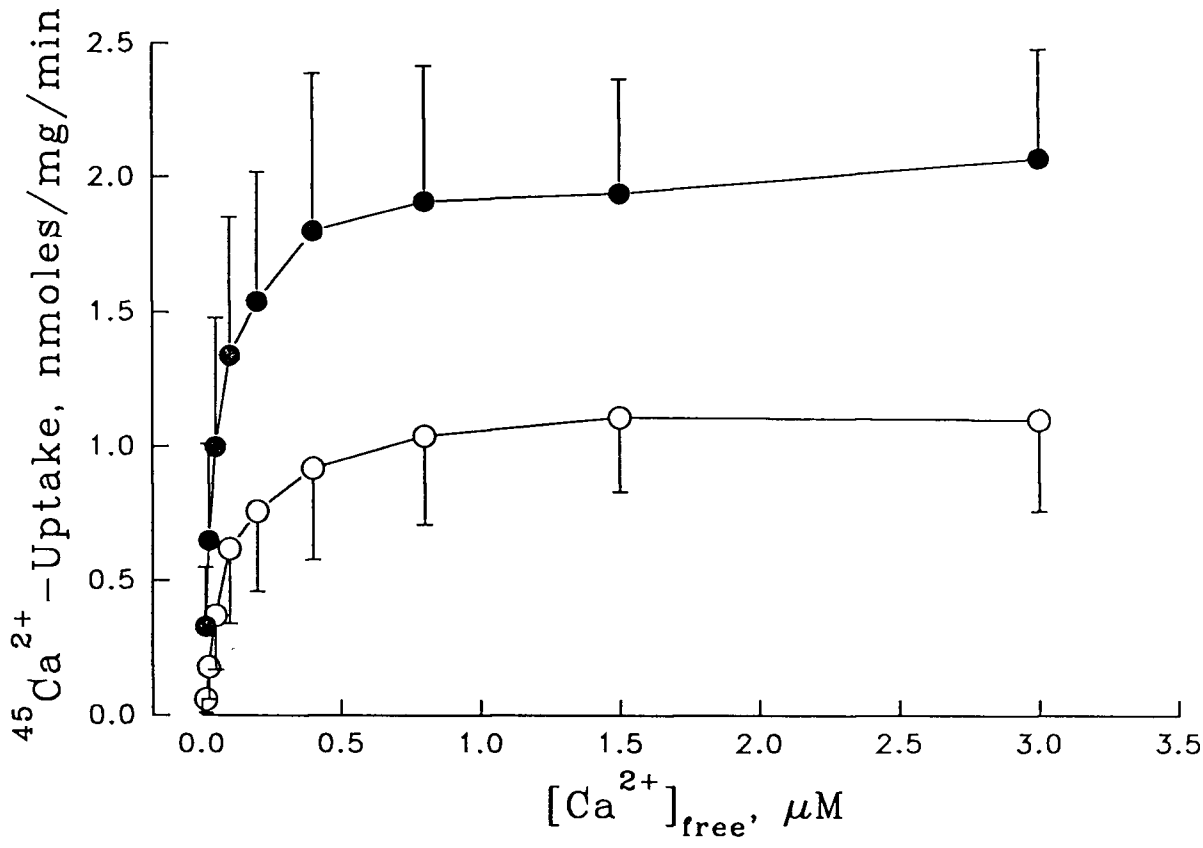

Figure 20. Effect of the catalytic subunit of protein kinase A on Ca^{2+} activation of $^{45}Ca^{2+}$ uptake. The results represent the mean ± S.E.M. for three experiments. ○ - Control, ● - 400 units/ml C-subunit.

Table III. Effect of protein kinase inhibitors on $^{45}Ca^{2+}$ uptake in plasma membrane vesicles. The transport of Ca^{2+} was measured in the presence of 0.5 μM free Ca^{2+} and 5 mM Mg^{2+}. The results represent mean ± S.E.M. for three experiments except where indicated within parentheses.

	$^{45}Ca^{2+}$-Uptake (nmoles/mg/min)	
	Basal	ATP-dependent
Control	0.08 ± 0.01	0.35 ± 0.02
CGS9343B (10 μM)	0.08 ± 0.01	0.32 ± 0.02
CGP 41 (0.5 μM)	0.08 ± 0.01	0.34 ± 0.04
HA1004 (10 μM)	0.08 ± 0.01	0.34 ± 0.03
C-subunit (300U/ml)	0.08 ± 0.01(4)	0.88 ± 0.12(4)*
C-subunit + HA1004	0.08 ± 0.01(4)	0.87 ± 0.10(4)*

*significantly different from Control ($P<0.01$)

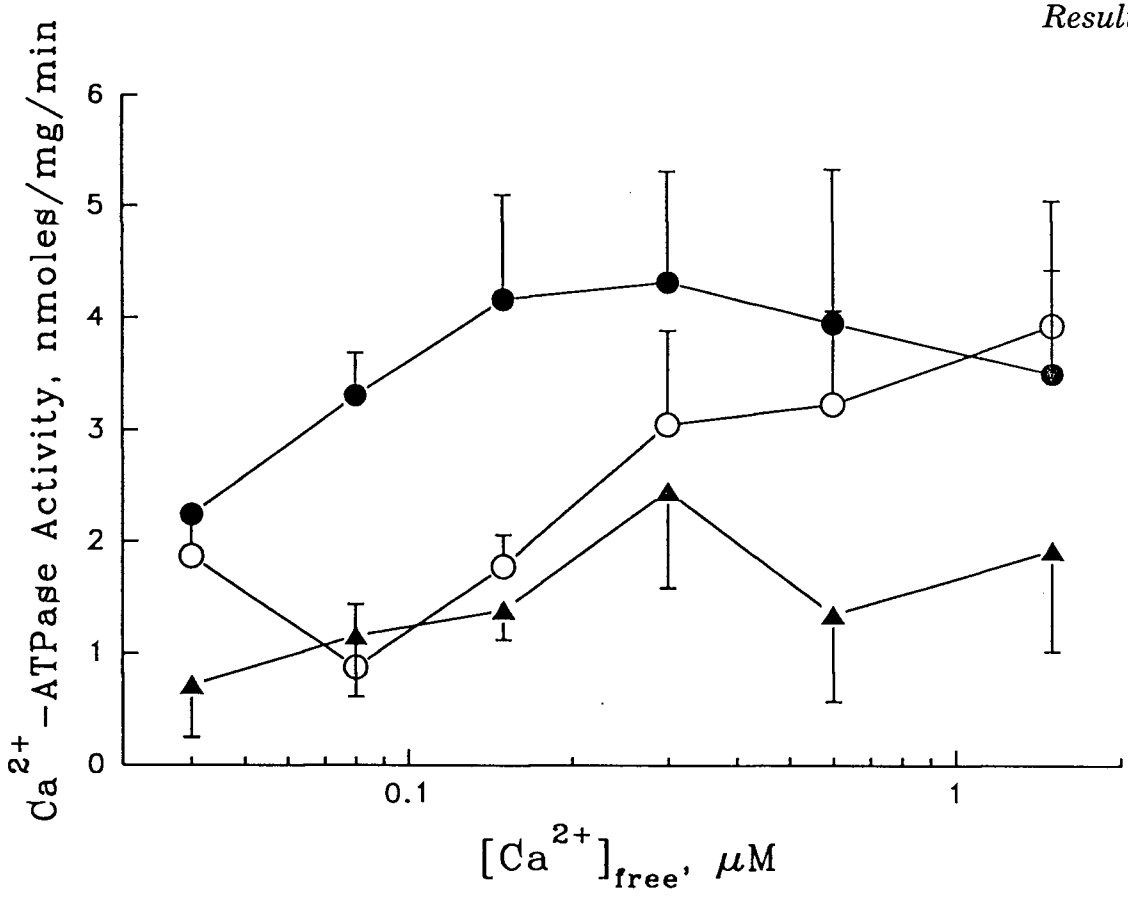

Figure 21. Effect of catalytic subunit of protein kinase A and the inhibitor HA 1004 on Ca^{2+}-dependent ATPase activity. The Mg^{2+} concentration was 0.5 mM. The results represent the mean ± S.E.M. for three experiments. ○ - Control, ● - 300 units/ml C-subunit, ▲ - 10 μM HA 1004.

Both TFP and compound 48/80 were found to inhibit $^{45}Ca^{2+}$-uptake activity in a concentration-dependent manner (Figs. 22 and 23). The inhibitory effects were observed with as little as 10 μM TFP (Fig. 22) and 10 μg/ml compound 48/80 (Fig. 23). TFP completely inhibited Ca^{2+} transport activity at 100 μM, while compound 48/80 produced almost complete inhibition at 1 mg/ml.

The effects of these agents on Ca^{2+}-ATPase activity were somewhat more complex; TFP did not significantly inhibit the Ca^{2+}-dependent ATPase activity at two different Ca^{2+} concentrations (Fig. 24). Inhibition of Ca^{2+}-dependent ATPase activity by compound 48/80 was less consistent when tested at 3 different Ca^{2+} concentrations (Fig. 25). This activity was significantly inhibited by 1 μg/ml of compound 48/80 at all Ca^{2+} concentrations. Ten μg/ml did not affect the Ca^{2+}-dependent ATPase, while 100 μg/ml only inhibited it at 1.0 μM free Ca^{2+}. It was very interesting to observe that compound 48/80 reproducibly stimulated "Basal" (Mg^{2+}-ATPase) and "Total" ATPase activity (the activity in the presence of both Ca^{2+} and Mg^{2+}) in a concentration-dependent manner (Fig. 26).

A supposedly more potent inhibitor of CaM, CGS 9343B (Norman et al., 1987), produced no inhibition of Ca^{2+}-uptake (Table III) or Ca^{2+}-dependent ATPase (Table IV) activities in our preparations.

Determination of Endogenous CaM

To determine whether guinea-pig pancreatic acinar plasma membrane preparations contained endogenous CaM, membranes were boiled in a medium containing 0.2 mM EDTA and the extracts were then assayed for the presence of CaM. The indicator system used to detect CaM within guinea-pig pancreatic acinar plasma membrane extracts was erythrocyte plasma membrane Ca^{2+}-ATPase, an enzyme stimulated by CaM. Figure 27 shows the results from a

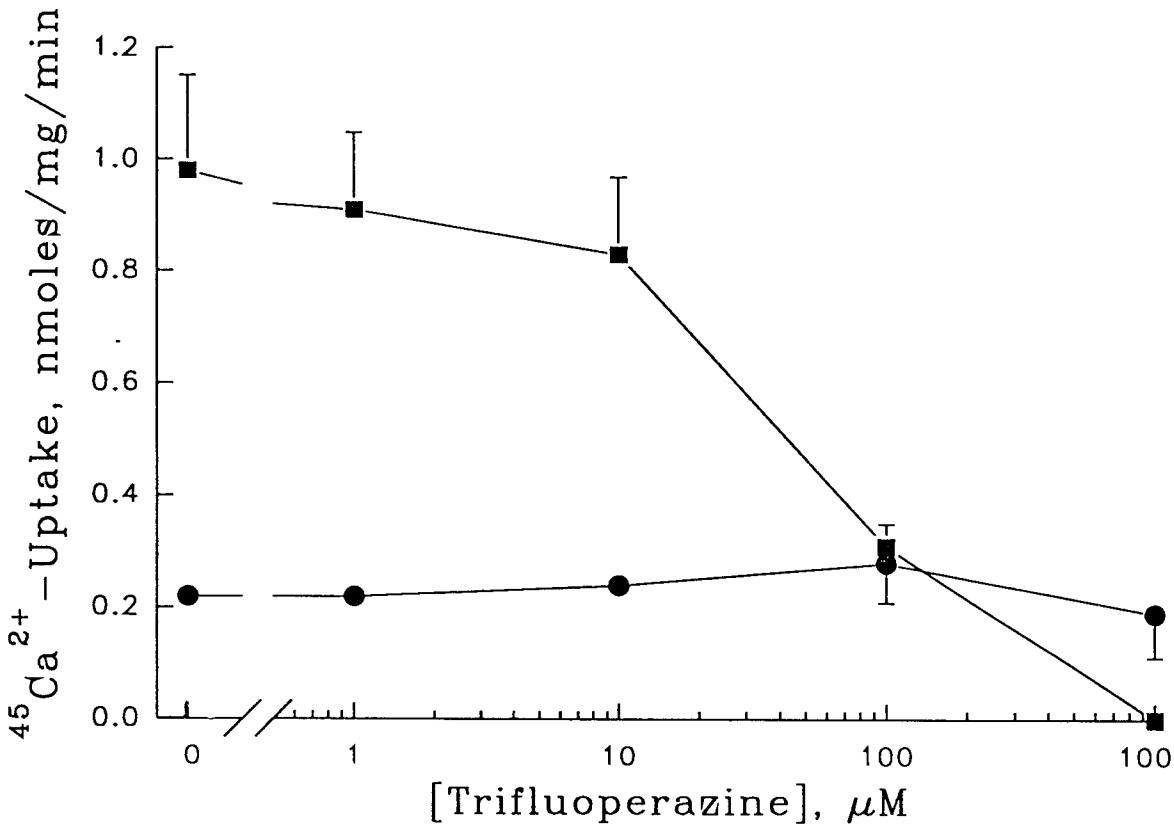

Figure 22. Effect of trifluoperazine on $^{45}Ca^{2+}$ uptake in guinea-pig pancreatic acinar plasma membrane vesicles. The transport of Ca^{2+} was measured as described in the **METHODS** in the presence of 1.5 μM free Ca^{2+} and 5 mM Mg^{2+}. The results represent the mean ± S.E.M. of three experiments. ● - Basal, ■ - ATP-dependent uptake.

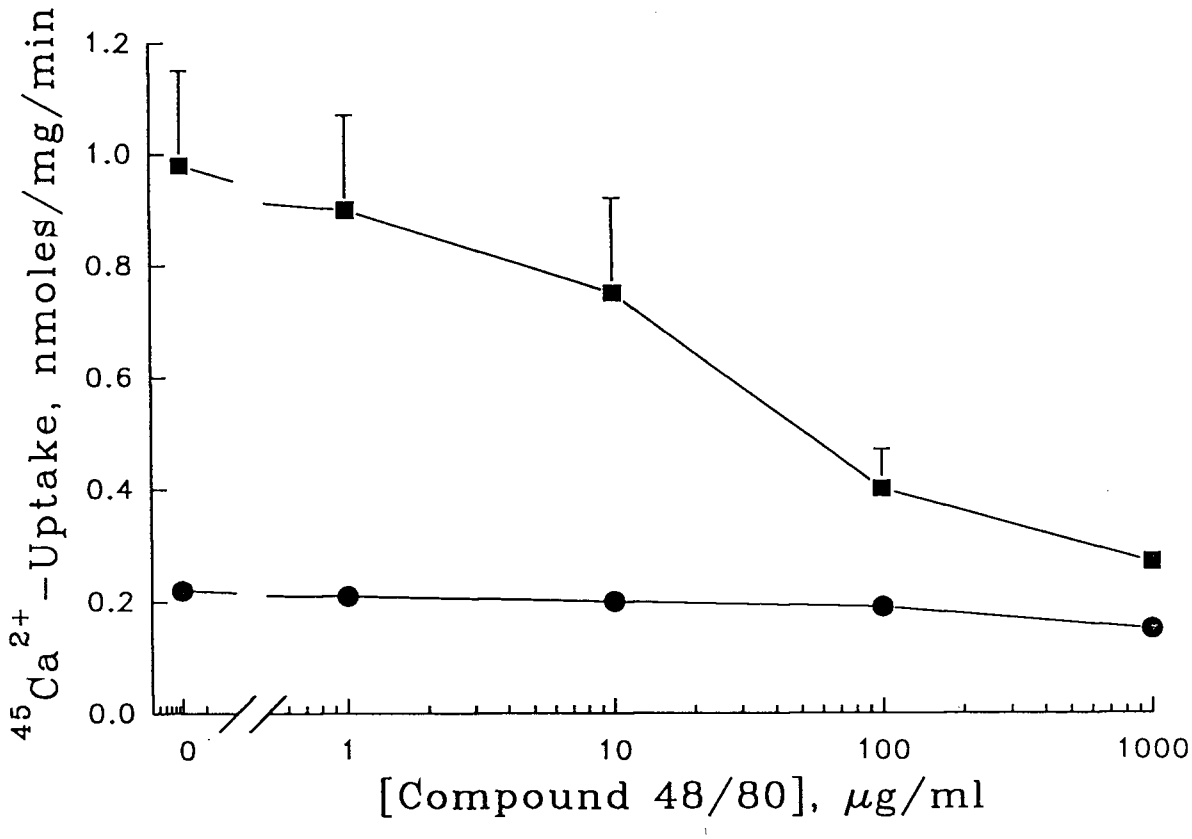

Figure 23. Effect of compound 48/80 on $^{45}Ca^{2+}$ uptake in guinea-pig pancreatic acinar plasma membrane vesicles. The transport of Ca^{2+} was measured as described in the **METHODS** in the presence of 1.5 μM free Ca^{2+} and 5 mM Mg^{2+}. The results represent the mean ± S.E.M. of three experiments. ● - Basal, ■ - ATP-dependent uptake.

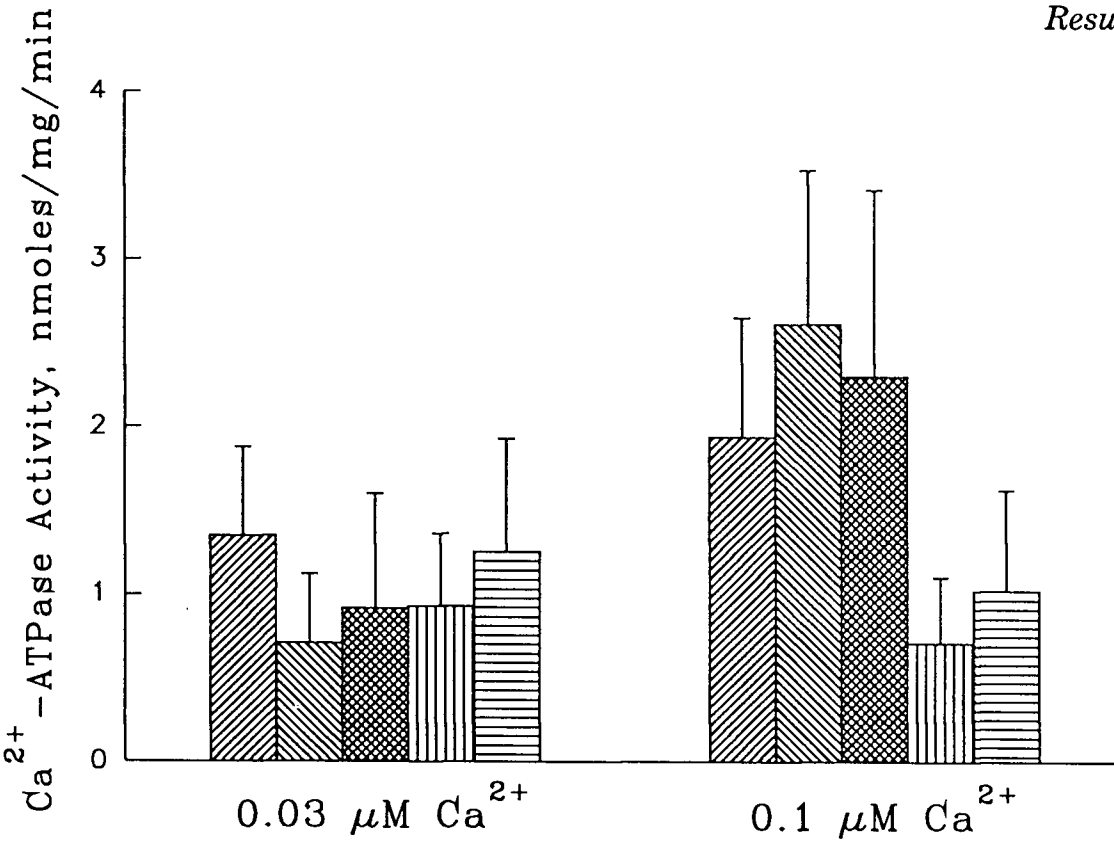

Figure 24. Effect of trifluoperazine (TFP) on Ca^{2+}-dependent ATPase activity at different Ca^{2+} free concentrations. The Mg^{2+} concentration was 0.5 mM. The results represent the mean ± S.E.M. for four experiments. ▨ - Control, ▨ - 1.0 μM TFP, ▨ - 10 μM TFP, ▨ - 100 μM TFP, ▨ - 1 mM TFP.

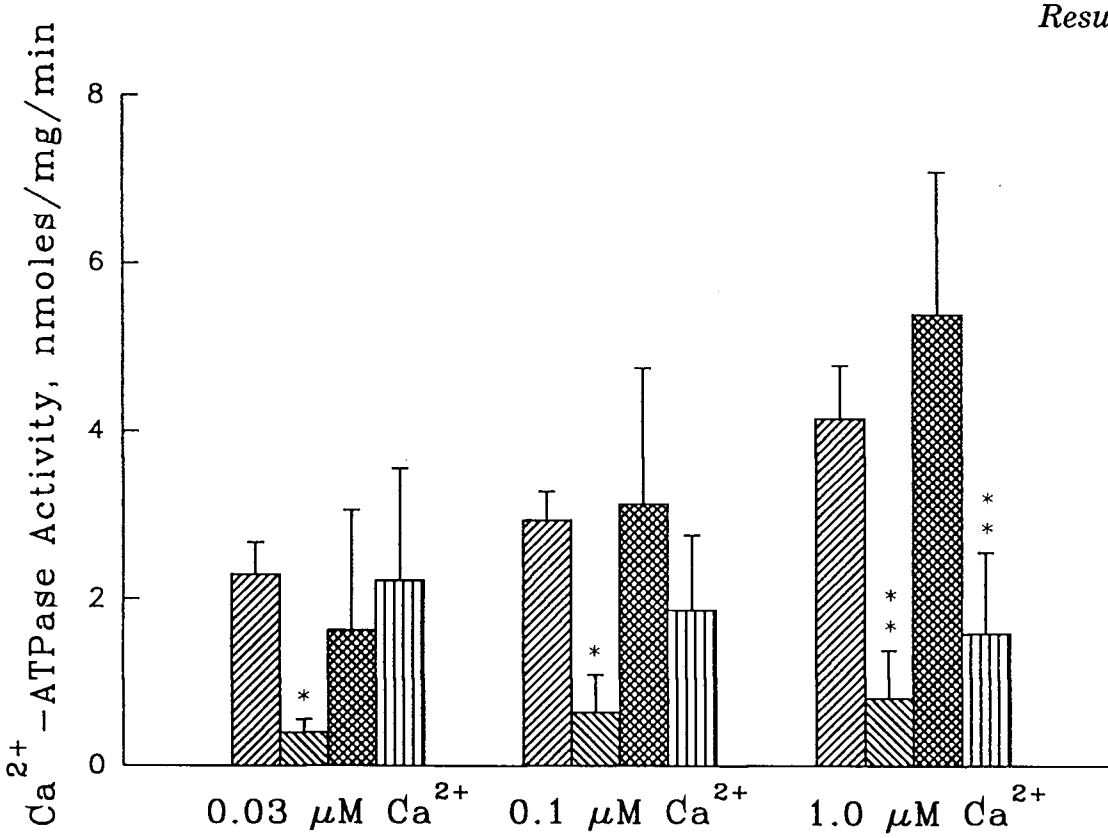

Figure 25. Effect of compound 48/80 on Ca^{2+}-dependent ATPase activity at different Ca^{2+} free concentrations. The Mg^{2+} concentration was 0.5 mM. The results represent the mean ± S.E.M. for four experiments. ▨ - Control, ▨ - 1.0 μg/ml compound 48/80, ▨ - 10 μg/ml compound 48/80, ▨ - 100 μg/ml compound 48/80. * - significantly different from Control at $P<0.005$, ** - significantly different from Control at $P<0.05$.

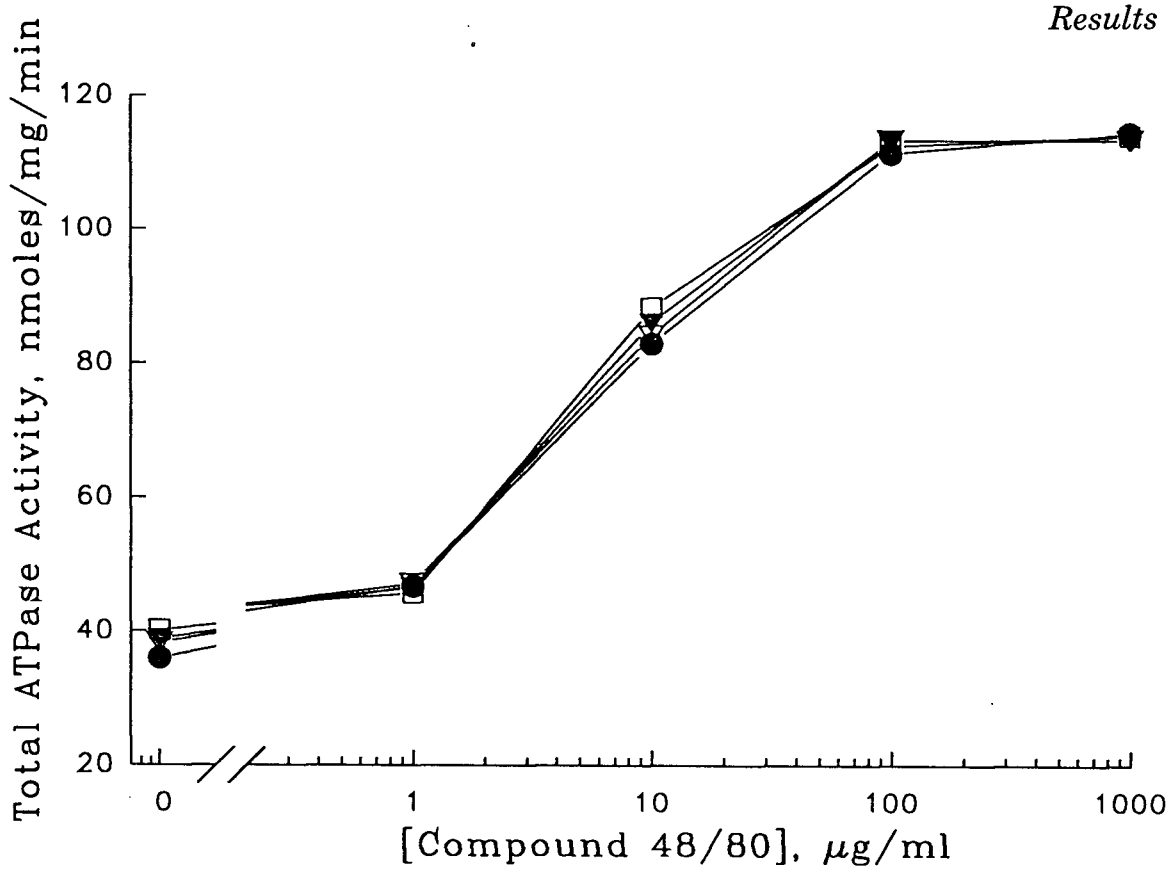

Figure 26. Effect of compound 48/80 on total ATPase activity at different Ca^{2+} free concentrations and in the presence of 0.5 mM Mg^{2+}. The results represent the mean of four experiments. ● - 0 Ca^{2+} (Basal activity), ∇ - 0.03 μM Ca^{2+}, ▼ - 0.1 μM Ca^{2+}, □ - 1.0 μM Ca^{2+}.

Table IV. Effect of inhibitors CGS 9343B and CGP 41 251 on Ca^{2+}-dependent ATPase activity at different Ca^{2+} concentrations. The results represent mean ± S.E.M. of three experimens.

	Ca^{2+}-ATPase Activity (nmoles/mg/min)		
μM Ca^{2+}	Control	CGS	CGP
0.08	3.40 ± 1.06	4.11 ± 1.25	4.11 ± 1.11
0.30	3.56 ± 0.74	4.25 ± 1.13	4.01 ± 0.50
1.50	4.09 ± 1.16	4.90 ± 0.89	3.88 ± 0.71

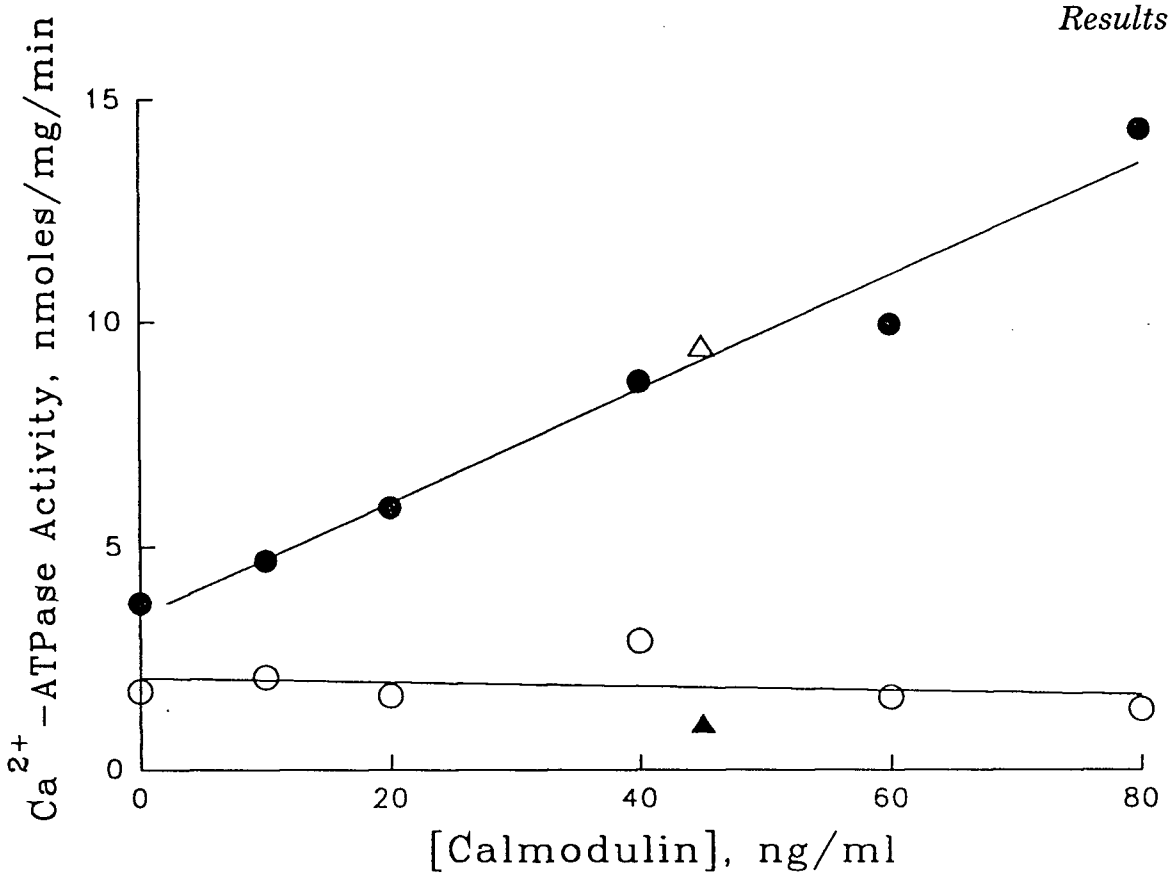

Figure 27. Stimulation of erythrocyte Ca^{2+}-ATPase activity by exogenous CaM. The experiments were carried out in the presence of 1.0 μM free Ca^{2+} and 1.0 mM Mg^{2+} and in the absence (●) or presence (○) of 60 μM trifluoperazine (TFP). Superimposed on these curves are resultant activities measured when erythrocyte membranes were treated with boiled guinea-pig pancreatic acinar plasma membrane extracts in the absence (Δ) and presence (▲) of TFP. These data are results of a representative experiment.

representative experiment. Exogenous CaM produced a concentration-dependent stimulation of erythrocyte Ca^{2+}-ATPase activity. This red cell marker activity was also stimulated by guinea-pig pancreatic acinar plasma membrane extracts. Stimulation by both the exogenous CaM and plasma membrane extracts was blocked by 60 μM TFP.

Removal of Endogenous CaM

Attempts were made to deplete the endogenous CaM so that possible stimulation by exogenous CaM could be determined and CaM affinity chromatography could be utilized for the purification of Ca^{2+}-ATPase. Hypertonic-hypotonic treatment in the presence of EGTA, as described by Caroni and Carafoli (1981a), proved unsuccessful in removing endogenous CaM from plasma membranes (not shown). A promising strategy utilizing Sephadex G-75 column chromatography of detergent-solubilized plasma membrane proteins was also attempted for depletion of endogenous CaM. As shown in figure 28, proteins were eluted from the column in 2 peaks. As expected, when column eluates were analyzed by SDS-PAGE, the first peak contained high molecular weight proteins, while the second peak represented smaller proteins close in size to CaM (not shown). Enzymatic assay of these fractions was precluded by the presence of high concentrations of Triton X-100 arising from the solubilization and equilibration media.

Regulation by Protein Kinase C

It has been suggested that PKC is involved in the stimulation of Ca^{2+} efflux from pancreatic acinar cells (Muallem et al., 1988b). In our studies, 70 μg/ml purified PKC from rat brain and 1.0 μM of the phorbol ester TPA failed to stimulate Ca^{2+}-uptake, in the absence or presence of 80 μg/ml

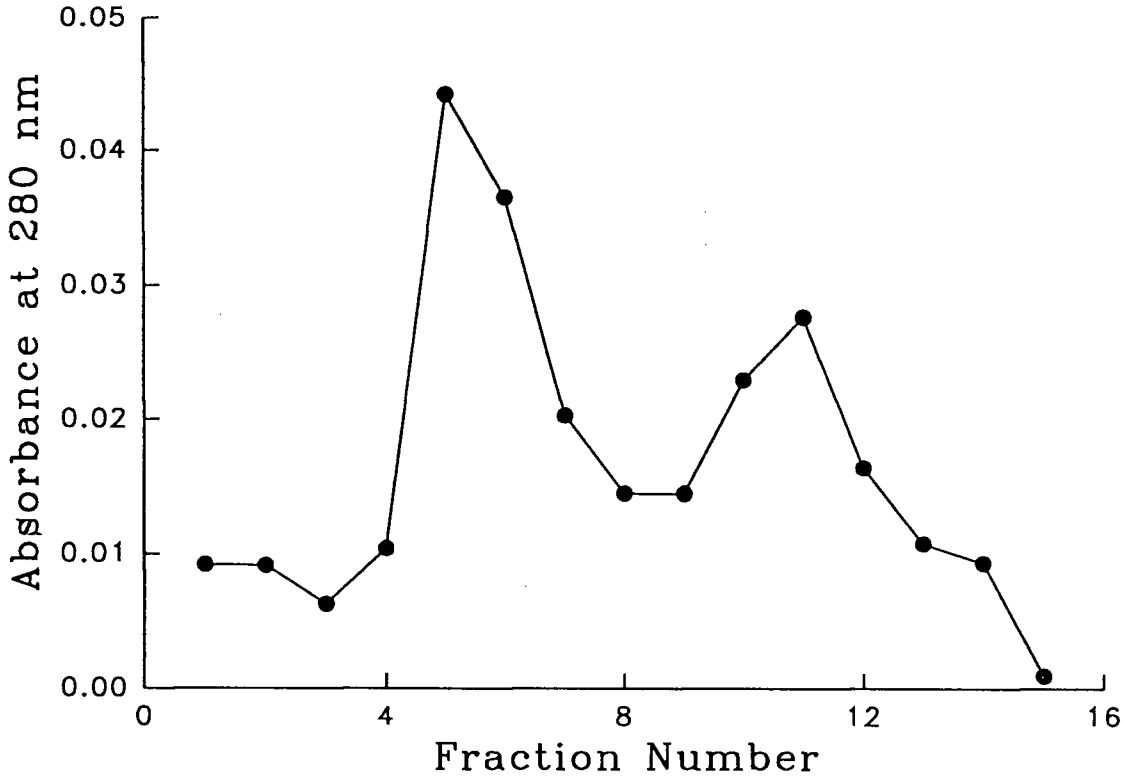

Figure 28. Protein profile (absorbance at 280 mm) of fractions eluted from a Sephadex G-75 chromatography column after applying solubilized guinea-pig pancreatic acinar plasma membranes. The elution medium was identical to the solubilization buffer described in **METHODS**.

phosphatidylserine (PS) (Fig. 29). PS alone also produced no significant effect on this activity (Fig. 29) or on the Ca^{2+}-ATPase activity (Table V). Both PKC and TPA were unable to affect the PS-stimulated Ca^{2+}-ATPase activity. However, a combination of PS and TPA produced a significant stimulation of control Ca^{2+}-ATPase activity at both Ca^{2+} concentrations tested, whereas the PS and PKC combination produced stimulation only at 1.0 μM free Ca^{2+}. Another activator of PKC, 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SA-DG), failed to affect either activity in the presence of PS at low Ca^{2+} concentration (Fig. 30 and Table V), but it significantly stimulated Ca^{2+}-ATPase at 1.0 μM Ca^{2+} (Table V).

CGP 41 251, a potent and selective inhibitor of PKC (Meyer et al., 1989), did not inhibit Ca^{2+}-transport (Table III) or Ca^{2+}-ATPase (Table IV) activities.

Regulation by Inositol Phosphates

The effects of 1 μM inositol 1,4,5-trisphosphate (IP_3) and 5 μM inositol 1,3,4,5-trisphosphate (IP_4) were studied on Ca^{2+}-transport only. Figure 31 shows that neither the individual nor a combination of inositol phosphates produced a statistically significant effect on Ca^{2+}-uptake.

SOLUBILIZATION AND PURIFICATION OF Ca^{2+}-ATPase

The presence of endogenous CaM in our preparations and the previous demonstration of a 133 kDa CaM-binding protein in rat pancreatic acinar plasma membranes (Ansah et al., 1984) suggested that this protein may have a direct role in stimulating the Ca^{2+}-ATPase or Ca^{2+} transport activities of guinea-pig pancreatic acinar plasma membrane, in a manner similar to that seen in erythrocytes. The possibility of a direct interaction between CaM and the ATPase indicated that CaM-affinity chromatography might be utilized in an attempt to purify the enzyme.

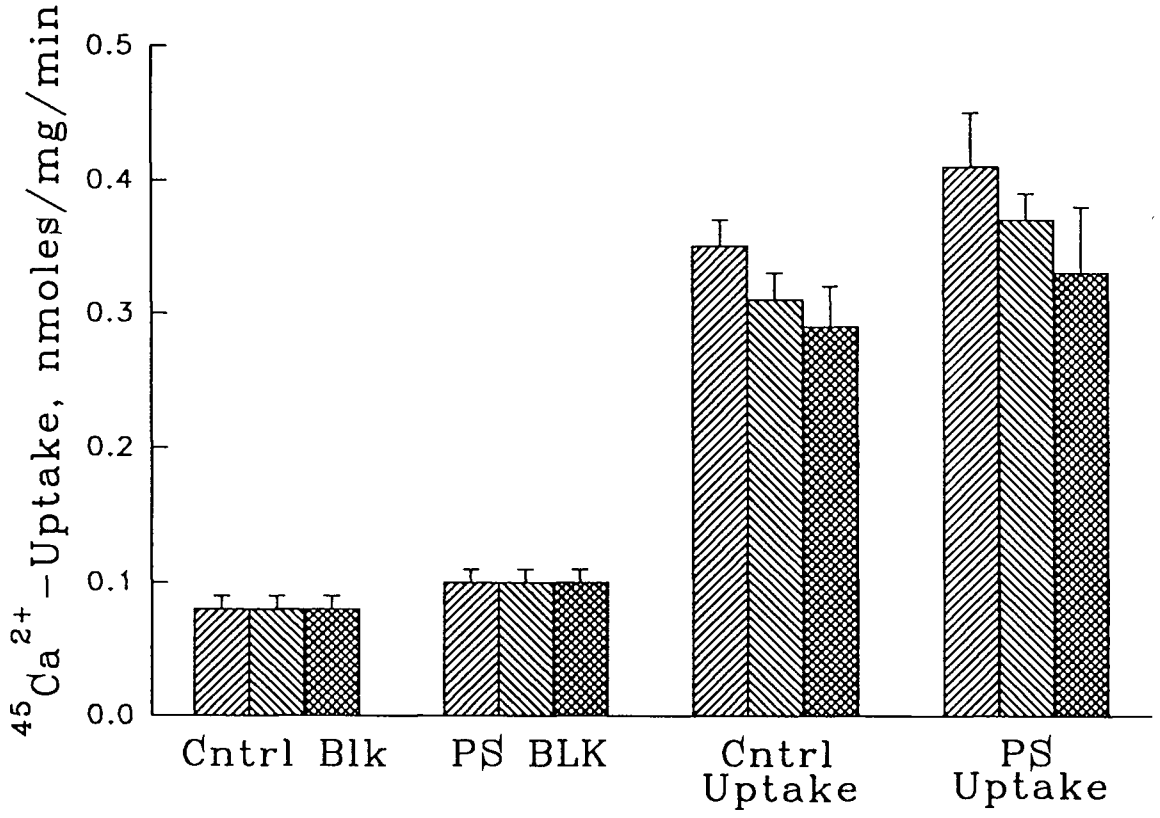

Figure 29. Effect of Protein kinase C or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) on $^{45}Ca^{2+}$ uptake by guinea-pig pancreatic acinar plasma membrane vesicles. Experiments were carried out at 0.5 μM free Ca^{2+}. The concentration of PS used was 80 μM/ml. The results represent the mean ± S.E.M. for three experiments. ▨ - Control, ▧ - 70 μg/ml PK-C, ▩ - 1.0 μM TPA. Cntrl Blk - control basal activity, PS Blk - basal activity in the presence of phosphatidylserine, Cntrl Uptake - ATP-dependent uptake in the absence of phosphatidylserine, PS Uptake - ATP-dependent uptake in the presence of phosphatidylserine.

Table V. Effect of Protein kinase C, SA-DG and TPA on Ca^{2+}-dependent ATPase activity of guinea-pig pancreatic acinar plasma membranes. The effects of PKC, SA-DG and TPA were studied in the presence of PS. The results represent mean ± S.E.M. of three experiments.

	Ca^{2+}-ATPase Activity (nmoles/mg/min)	
	0.1 μM Ca^{2+}	1.0 μM Ca^{2+}
Control	2.73 ± 1.02	3.36 ± 0.85
PS (80 μg/ml)	4.98 ± 1.88	7.11 ± 1.63
PK-C (70 μg/ml)	6.59 ± 2.68	8.21 ± 2.02*
SA-DG (8 μg/ml)	4.70 ± 1.32	8.27 ± 0.85**
TPA (1.0 μM)	9.34 ± 1.31*	13.38 ± 3.51*

*Significantly different from Control ($P<0.05$)

**Significantly different from Control ($P<0.01$)

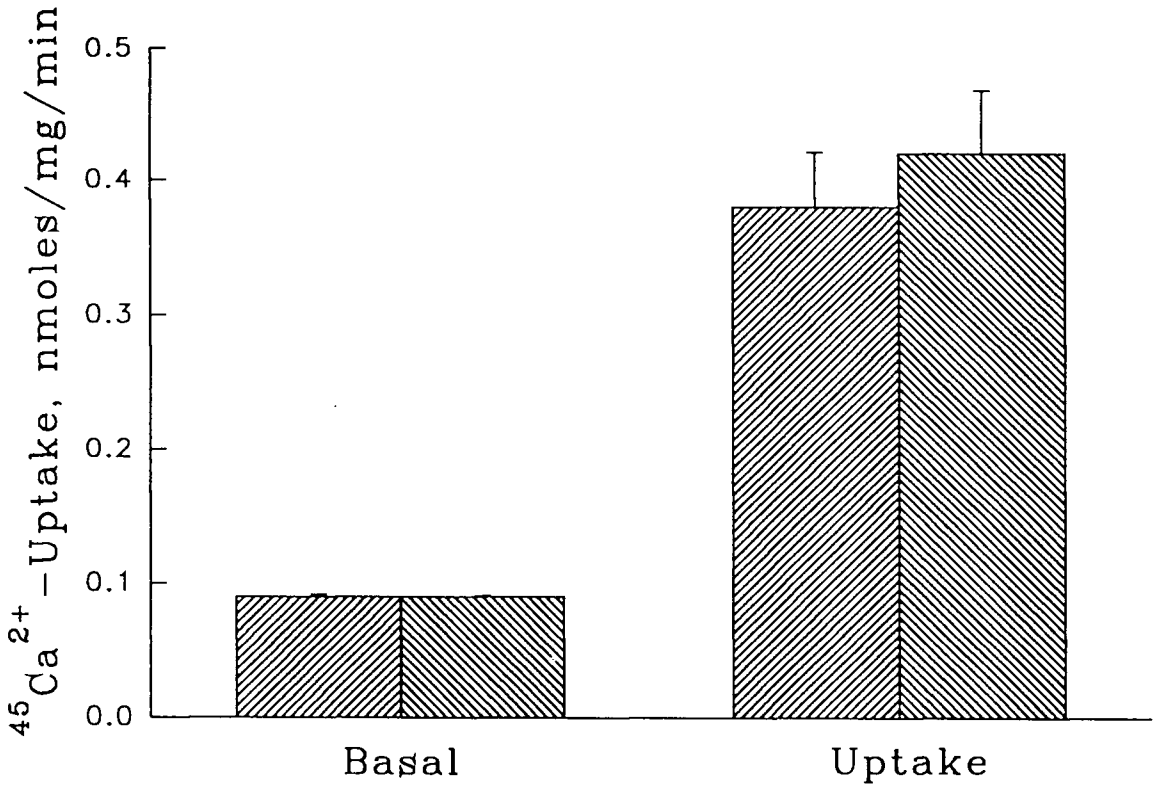

Figure 30. Effect of 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SA-DG) on $^{45}Ca^{2+}$ uptake. The results represent the mean ± S.E.M. for three experiments. Experiments were carried out in the presence of 0.5 μM free Ca^{2+} and 80 μg/ml phosphatidylcholine. ▨ - Control, ▨ - 8 μg/ml SA-DG.

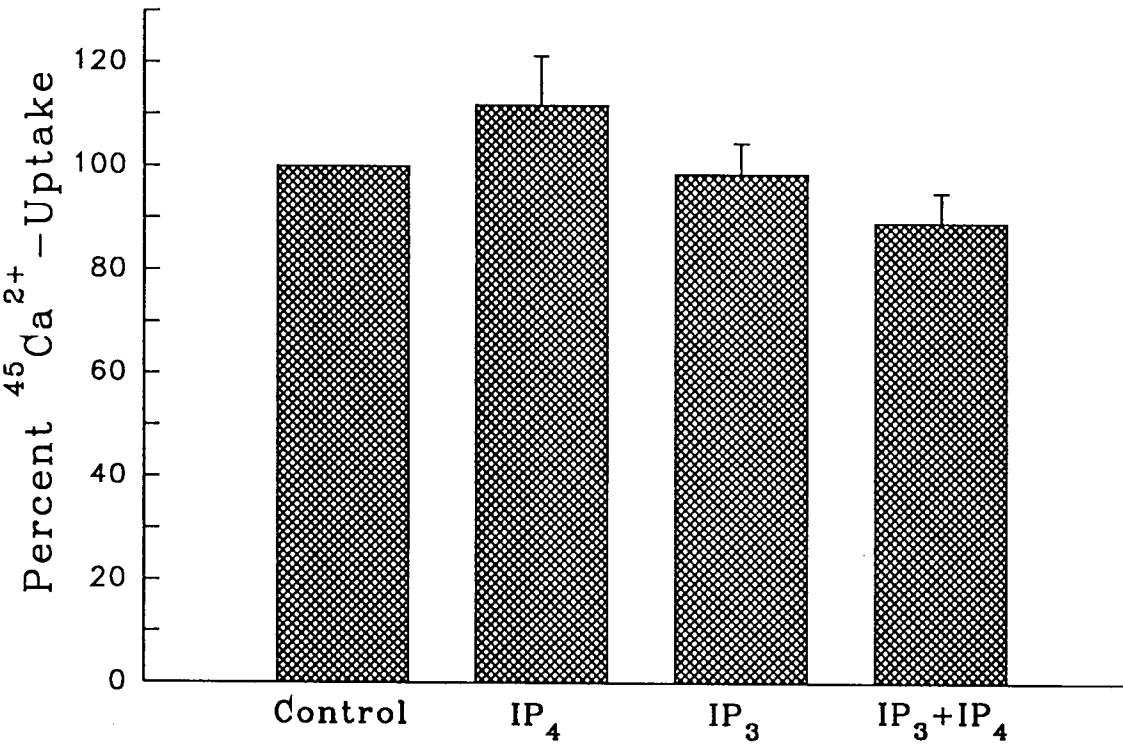

Figure 31. Effect of 5 μM IP_4 and 1 μM IP_3 on $^{45}Ca^{2+}$-uptake in guinea-pig pancreatic acinar plasma membranes. The results represent the mean ± S.E.M. for three experiments.

In early studies, we were able to separate two major proteins from the solubilized guinea-pig pancreatic acinar plasma membranes using the CaM-affinity column. However, it was discovered that most of the ATPase activity, comprising the greater part of the applied protein, did not bind to the column, but came out in the void volume (Fig. 32). Further attempts to increase the protein binding to the column and optimize purification proved unsuccessful. However, careful analysis of the eluted fractions by SDS-PAGE and ATPase assay was carried out. The following is a brief summary of some of the strategies used and the results obtained.

1. Attempts at removing solubilized endogenous CaM by using Sephadex G-75 prior to applying the solubilizate to a CaM-agarose column failed to increase the binding of any proteins to the latter column.
2. A number of protease inhibitors, including Leupeptin, Pepstatin A, TPCK and PMSF were added to the buffers in an attempt to retard possible proteolysis of the CaM binding site of the enzyme by endogenous proteases. This strategy too was unproductive in increasing the protein binding to CaM-agarose or the ATPase activity in the eluted fractions.
3. Excluding Asolectin (an acidic phospholipid-rich mixture) from buffers or substituting it with phosphatidylcholine did not appear to increase the binding of proteins or ATPase activity in fractions eluted from the CaM-affinity column. In another attempt to increase the yield of eluted proteins, cruder starting material (P_3 instead of 27/35% sucrose interface) was used. This procedure resulted in broader bands observed in the polyacrylamide gels. However, it failed to produce any new bands or increase the ATPase activity in eluted fractions.
4. To overcome the problem of low binding to CaM-agarose, we tested Cibacron Blue 3GA-agarose and Reactive Red 120-agarose affinity columns.

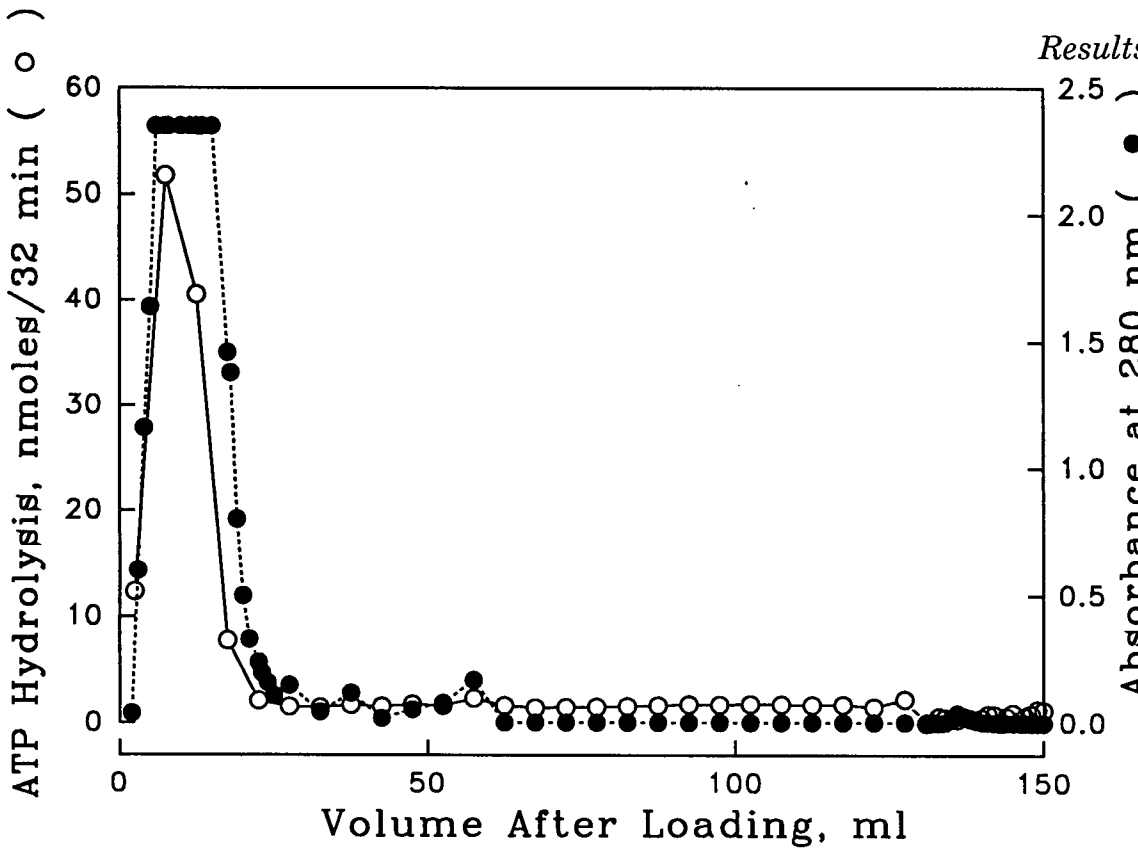

Figure 32. ATPase activity and absorbance at 280 nm of fractions collected from CaM-agarose column following loading and washing. The proteins were solubilized using a method similar to that described by Caroni and Carafoli (1981a). The graph represents a typical experiment.

As seen with CaM-agarose, most of the protein and ATPase activity showed negligible retention by the column. However, we were able to specifically elute three major proteins from both columns using ATP in the elution buffer. Unfortunately, these proteins did not appear to represent the ATPase.

5. An attempt to use gel filtration (using Ultrogel) to separate the proteins by molecular size was unsuccessful. All solubilized plasma membrane proteins appeared to migrate together in the micelles and were eluted in the void volume or shortly thereafter.
6. The use of higher KCl concentration (0.5 M) and 0.6 M sucrose in the solubilization buffers, according to the method of Caroni and Carafoli (1981a), failed to increase binding. On the other hand, lowering the KCl concentration from 0.5 M to 0.1 M and compensating the resulting loss of ionic strength to some extent by increasing the buffer concentration to 100 mM from 20 mM HEPES, pH 7.4 produced somewhat improved binding. However, the ATPase activity and the protein yield were too small to allow further characterization (Fig. 33).

EXPERIMENTS WITH PLASMA MEMBRANES FROM HUMAN PANCREAS

The human pancreatic tissue was obtained from the Pacific Organ Retrieval for Transplantation program. The experiments depended on the availability of tissue. Hence, only a few experiments were possible. It was found that the tissue was very difficult to digest with collagenase to obtain the acini. Preliminary experiments showed that plasma membranes prepared from human pancreatic acini were somewhat more buoyant than those from guinea-pigs. This was illustrated by an enrichment of the plasma membrane marker Na^+/K^+-

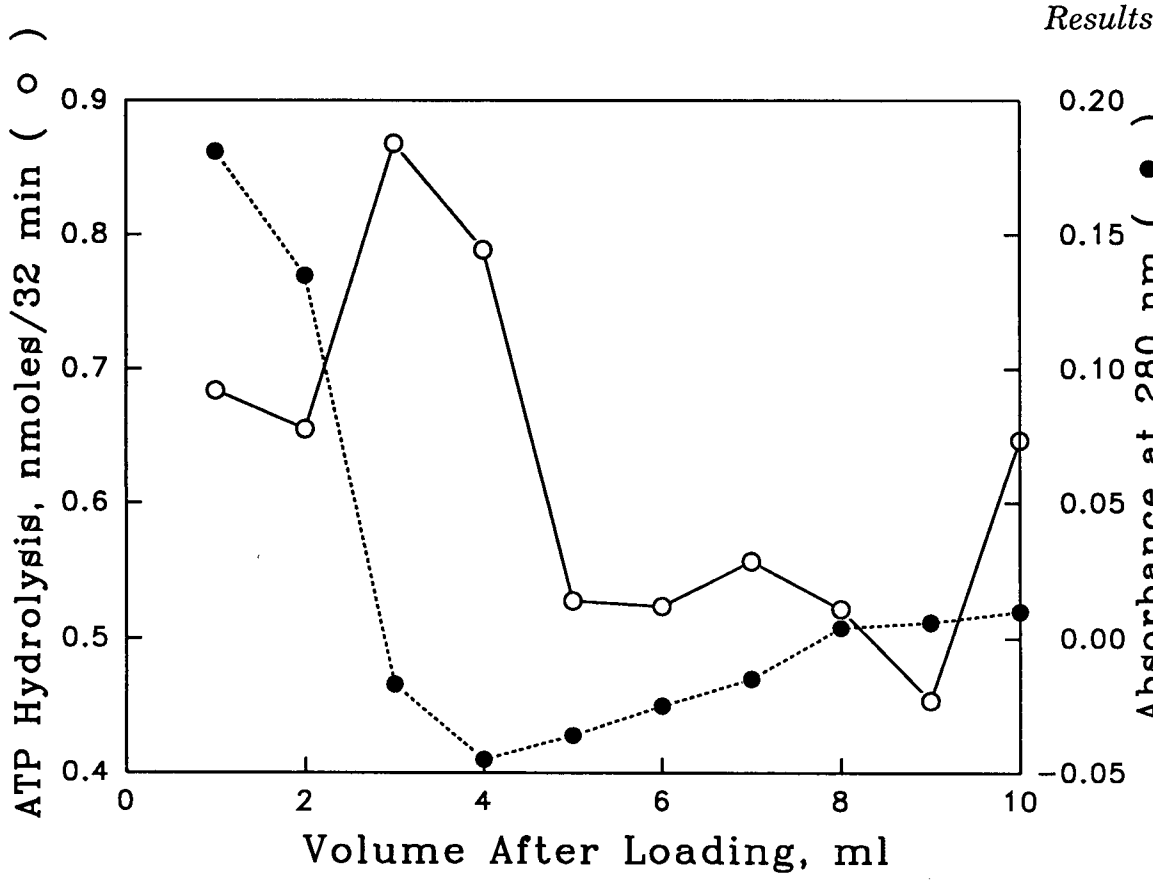

Figure 33. ATPase activity and absorbance at 280 nm of fractions eluted from the CaM-agarose affinity column. The solubilization medium was modified by lowering KCl concentration to 100 mM and raising HEPES concentration to 100 mM. The activity was measured in the presence of 1 mM $MgCl_2$ and 1 μM free Ca^{2+}. The graph represents a typical experiment.

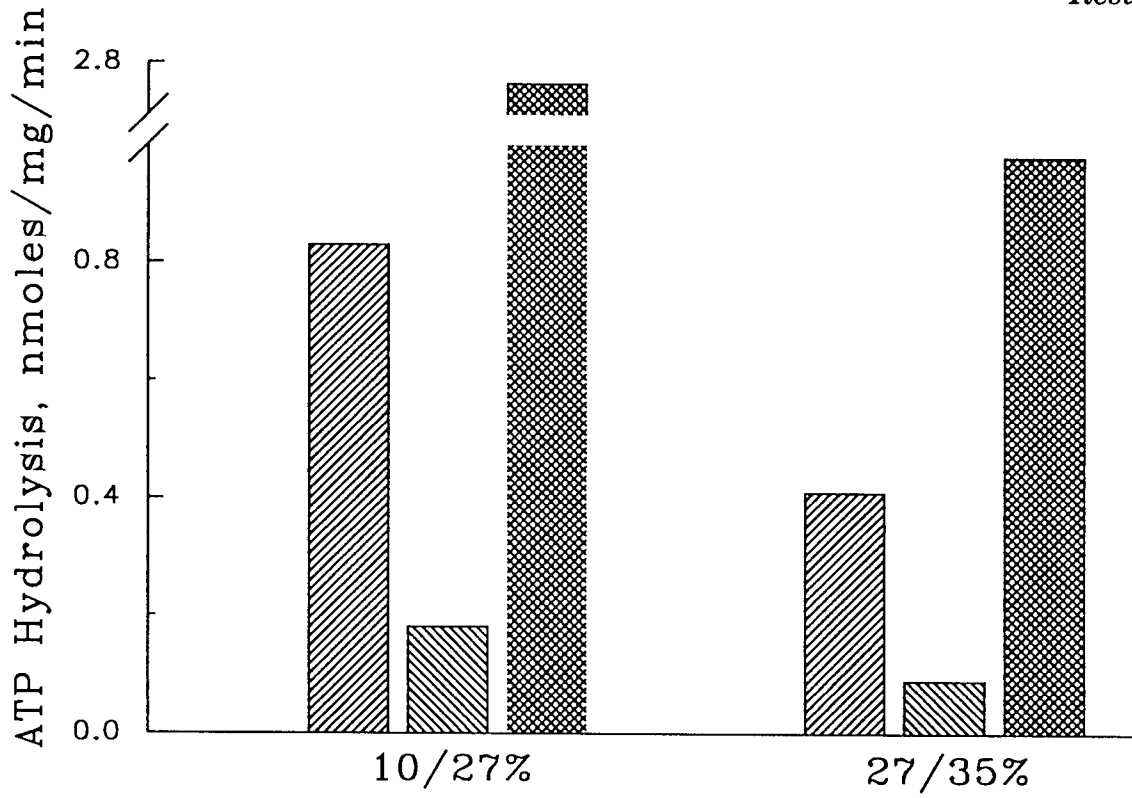

Figure 34. Enrichment of Ca^{2+}-dependent ATPase and Na^+/K^+-ATPase activities in human pancreatic acinar plasma membranes. Experiments were carried out at 1.0 mM free Ca^{2+}. ▨ - Ca^{2+}-ATPase activity in the absence of Mg^{2+}, ▨ - Ca^{2+}-ATPase activity in the presence of 0.5 mM $MgCl_2$, ▨ - Na^+/K^+-ATPase activity. The results represent a typical experiment.

ATPase in the 10/27% sucrose density interface (Fig. 34). A Ca^{2+}-dependent ATPase activity was also observed in this fraction. In other experiments, CaM was found to stimulate the Ca^{2+}-ATPase activity more than 5-fold at low Ca^{2+} (Fig. 35). Trifluoperazine alone had no effect on Ca^{2+}-ATPase activity. However, it completely inhibited the stimulation by CaM (not shown). At higher free Ca^{2+} concentration, neither agent produced an effect on the Ca^{2+}-dependent ATPase activity.

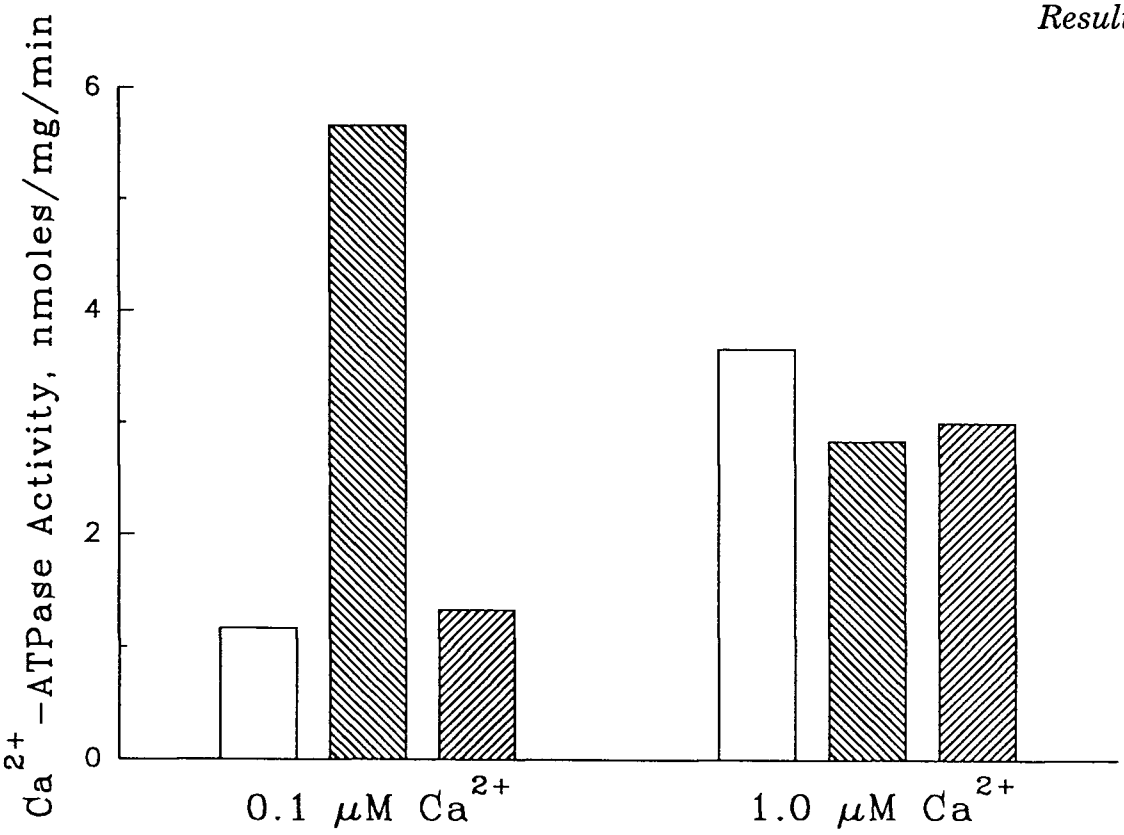

Figure 35. Effect of CaM and TFP on Ca^{2+}-dependent ATPase in human pancreatic acinar plasma membranes. ▭ - Control, ▨ - 6 μg/ml CaM, ▨ - 60 μM TFP. The results represent a typical experiment.

DISCUSSION

Previous efforts to characterize pancreatic acinar plasma membrane Ca^{2+}-ATPase have centered on the use of rat tissue. However, this species shows high levels of diphosphohydrolase (Ansah et al., 1984; LeBel et al., 1980), making characterization of the high affinity Ca^{2+}-ATPase difficult. In an attempt to find an alternative source of pancreatic plasma membranes with little or no diphosphohydrolase activity, both bovine and guinea-pig pancreata were investigated. Plasma membranes from bovine pancreas showed up to a 12-fold enrichment of Na^+/K^+-ATPase and Ca^{2+}-ATPase activities (data not shown). However, this tissue source was abandoned due to problems with bacterial contamination, difficulties with tissue digestion and low cellular viability. Although the guinea-pig pancreatic acinar plasma membranes displayed a low affinity non-specific ATPase activity similar to the previously reported diphosphohydrolase, we were still able to reproducibly demonstrate the presence of a high affinity Ca^{2+}-ATPase activity. In addition, the presence of a high affinity Ca^{2+}-transport activity in pancreatic acinar plasma membranes was demonstrated in this species. This species was therefore chosen for our studies.

The plasma membrane preparation showed a good enrichment of the plasma membrane marker, Na^+/K^+-ATPase. Since the plasma membrane of the pancreatic acinar cell has been reported to account for only 4.8% of the total membrane surface area (Bolender, 1974), a protein recovery of 1.3% in our preparations (Fig. 5), compared to the homogenate was considered good. This recovery also compared well with the plasma membrane yield (approximately 2% of the homogenate) from the preparative method of another laboratory (Bayerdörffer et al., 1985a)

BASIC CHARACTERIZATION

The ATP-dependent Ca^{2+}-transport activity present in our plasma membrane fraction required very low Ca^{2+} for activation (K_{Ca} = 0.04 μM) and was saturated at 1 μM free Ca^{2+} (Fig. 10). To our knowledge, a similar high affinity for Ca^{2+} has only been reported for the plasma membrane Ca^{2+}-ATPase of rat hepatocytes (Lin and Fain, 1984; Lotersztajn et al., 1981; Pavoine et al., 1987; Prpic et al., 1984), human hepatocytes (Lotersztajn et al., 1982) and human small intestine basolateral membranes (Kikuchi et al., 1988). The guinea-pig pancreatic acinar plasma membrane Ca^{2+}-pump, though, showed a 20-fold higher affinity for Ca^{2+} compared to that in rat tissue (Bayerdörffer et al., 1985a). Such a high affinity may suggest that the pump is active both in resting acinar cells, when the reported cytosolic free Ca^{2+} concentration ranges between 0.09 and 0.18 μM (Muallem et al., 1988b; Ochs et al., 1985; Powers et al., 1985) and during stimulation by secretagogues, when the free Ca^{2+} concentration approaches 0.8-1.3 μM (Muallem et al., 1988b; Pandol et al., 1985a; Powers et al., 1985). A role for the plasma membrane Ca^{2+}-pump in controlling intracellular Ca^{2+} at rest and during stimulation has been previously proposed for pancreatic acinar cells (Dormer et al., 1987; Muallem et al., 1988a, b, c, d). Most of these studies have been carried out using whole cells. The increase in intracellular free Ca^{2+} in response to stimulation by secretagogues was found to be transient (Muallem et al., 1988c; Pandol et al., 1985a; Streb and Schulz, 1983), and this might suggest that either the ER or plasma membrane Ca^{2+}-transporter or both are activated during stimulation. The rate of $^{45}Ca^{2+}$ efflux has been shown to increase in the presence of secretagogues (Muallem et al., 1988b). However, a rise in intracellular free Ca^{2+} concentration was not required for activation of the Ca^{2+}-pump (Muallem et al., 1988b).

The affinity of the guinea-pig pancreatic acinar plasma membrane Ca^{2+}-pump for Ca^{2+} was higher than that reported for the Ca^{2+}-pump of pancreatic acinar endoplasmic reticulum from rat (Bayerdörffer et al., 1985a; Brown et al., 1987) or guinea-pig (Galvan and Lucas, 1987). Rat pancreatic acinar plasma membrane Ca^{2+} transporter (Bayerdörffer et al., 1985a) and Ca^{2+}-activated, Mg^{2+}-dependent ATPase (Al-Mutairy and Dormer, 1985; Dormer and Al-Mutairy, 1987) had been reported to have a lower affinity for Ca^{2+} (K_{Ca} = 0.9 μM) than rat pancreatic acinar ER Ca^{2+}-ATPase (K_{Ca} = 0.16-0.17 μM) (Brown et al., 1987; Richardson and Dormer, 1984). Therefore, it was suggested that the plasma membrane Ca^{2+}-pump may not play a significant role in the maintenance of cytosolic free Ca^{2+} at rest (Al-Mutairy and Dormer, 1985; Dormer and Al-Mutairy, 1987). The high affinity of the plasma membrane Ca^{2+}-pump for Ca^{2+} in our preparations leads to a different conclusion. This property suggests that the enzyme may be involved in the fine regulation of intracellular Ca^{2+} in pancreatic acinar cells at rest, while the function of ER Ca^{2+}-pump may be the rapid sequestration of raised cytosolic Ca^{2+} to terminate the secretagogue signal.

The maximal rate of Ca^{2+}-transport in guinea-pig pancreatic acinar plasma membrane vesicles (0.83 nmoles/mg/min) compares well with that of rat panreatic acinar plasma membrane vesicles (Bayerdörffer et al., 1985a) and rat liver plasma membranes vesicles (Prpic et al., 1984), *viz.* 0.66 and 0.65 nmoles/mg/min, respectively.

Similar to the human erythrocyte Ca^{2+}-ATPase pump prototype, the guinea-pig pancreatic acinar plasma membrane Ca^{2+}-pump showed an apparent requirement for Mg^{2+} (Fig. 11). This Mg^{2+} requirement can be satisfied to a certain extent by endogenous Mg^{2+}, as has been shown previously for the Ca^{2+}-ATPase activities of plasma membranes prepared from rat pancreatic acini

(Ansah et al., 1984), rat liver (Lin and Fain, 1984; Lotersztajn et al., 1981), rat adipocytes (Pershadsingh and McDonald, 1980) and rat corpus luteum (Verma and Penniston, 1981). In our experiments, 25% of the maximal $^{45}Ca^{2+}$ transport into plasma membrane vesicles could be observed in the absence of added Mg^{2+}. CDTA, a cation chelator with higher affinity for Mg^{2+} than either EGTA or EDTA (Verma and Penniston, 1981), inhibited a major portion of this residual $^{45}Ca^{2+}$ uptake (Fig. 12). These results support the contention that sufficient endogenous Mg^{2+} is present in the plasma membrane preparations to support partial activation of the Ca^{2+}-pumping ATPase in the absence of added Mg^{2+}. However, for optimum stimulation of the pump, additional Mg^{2+} was required. Therefore, measurement of Ca^{2+}-dependent ATPase by others in the absence of added Mg^{2+} (see below) does not represent the optimal conditions for studying the Ca^{2+}-pumping activity.

The ATP hydrolytic activity displayed by guinea-pig pancreatic acinar plasma membrane preparations was complex. These preparations contained a low affinity ATPase activity which could be stimulated by either Ca^{2+} or Mg^{2+} (Fig. 14 and Fig. 15a, open circles). This activity appeared to be competitively inhibited by the other cation, as shown by the apparent inhibition of Ca^{2+}-dependent activity by increasing Mg^{2+} (Fig. 15a). Similar ATPase activities have previously been reported in plasma membranes from many rat tissues including pancreas (Forget and Heisler, 1976; Lambert and Christophe, 1978; Martin and Senior, 1980), liver (Lin and Fain, 1984; Lotersztajn et al., 1981), corpus luteum (Minami and Penniston, 1987), cardiac muscle (Anand-Srivastava et al., 1982; Zhao and Dhalla, 1988), osteosarcoma (Murray et al., 1983) and kidney cortex (Parkinson and Redde, 1971). In many cases, this Ca^{2+}(or Mg^{2+})-ATPase has been suggested to be an ecto-enzyme (Hamlyn and Senior, 1983; Lin, 1989; Lin and Russell, 1988; Tuana and Dhalla, 1988). Unlike

other ATPases, the liver ecto-ATPase has been shown to have a high-affinity for Ca^{2+} and Mg^{2+} (Lin and Russell, 1988). The Ca^{2+}-dependent and Mg^{2+}-dependent activities are not distinct, but appear to reside within the same protein (Lin, 1985).

In addition to the Ca^{2+}(or Mg^{2+})-ATPase, guinea-pig pancreatic acinar plasma membranes demonstrate a high affinity Ca^{2+}-dependent ATPase (K_{Ca} = 0.076 ± 0.022 μM) which was dependent on external Mg^{2+} (Fig 15b). In fact, this latter activity was only observed in the presence of high Mg^{2+} (0.5 mM or higher). A high affinity Ca^{2+}-ATPase has previously been reported in plasma membranes from a number of tissues including rat pancreas (Ansah et al., 1984; Dormer and Al-Mutairy, 1987; Ochs et al., 1988), liver (Iwasa et al., 1982) and corpus luteum (Minami and Penniston, 1987; Verma and Penniston, 1981). However, these other workers had been unable to demonstrate this activity in the presence of high, physiological Mg^{2+} concentrations. To overcome the difficulty in studying the high affinity Ca^{2+}-ATPase caused by the presence of an overwhelming low affinity Ca^{2+}(or Mg^{2+})-ATPase activity in their membrane preparations, some investigators have utilized low or zero added Mg^{2+} (Ansah et al., 1984; Minami and Penniston, 1987; Verma and Penniston, 1981). However, in our studies in guinea-pig pancreatic acinar plasma membranes, we failed to observe the high affinity Ca^{2+}-ATPase under these conditions. In the presence of a high Mg^{2+} concentration, the demonstration of high affinity Ca^{2+}-ATPase activity may depend on the assay conditions used. For example, in our experiments, where lower amounts of protein (16.7 μg/ml vs. 50 μg/ml) were used in a larger reaction volume (0.6 ml vs. 0.2 ml) and in which reactions were started with diluted membranes instead of ATP, the Ca^{2+}-dependent ATPase activity observed was not saturable and showed a 10-fold lower affinity for Ca^{2+} (data not shown).

The formation of a Ca^{2+}-dependent, hydroxylamine-sensitive phosphoprotein in guinea-pig pancreatic acinar plasma membranes (Fig. 18) also suggests the presence of a Ca^{2+}-pumping ATPase similar to that found within the erythrocyte plasma membrane. Phosphorylation of a 100,000 daltons protein has also been reported in other tissues and organelles such as corpus luteum plasma membranes (Minami and Penniston, 1987) and rat pancreatic acinar ER (Imamura and Schulz, 1985). The ER protein of Imamura and Schulz was also Ca^{2+}-dependent and hydroxylamine-sensitive. Although extensive ER marker studies were not performed, we found that the plasma membrane fraction of our preparations was not enriched in NADPH cytochrome c reductase (not shown), confirming the findings of Svoboda et al. (1976), whose modified method was followed. Furthermore, our preparation showed no stimulation of Ca^{2+}-dependent ATPase activity by potassium (Fig. 17), whereas the ER enzyme has been reported to be stimulated by this cation (Galvan and Lucas, 1987). Therefore, it seems unlikely that the protein phosphorylated in our preparations was from an ER contaminant. In addition to the 100,000 daltons phosphoprotein, a smaller protein (M_r 50,000) was also phosphorylated. However, this phosphoprotein was stable in hydroxylamine and appeared to be Mg^{2+}-dependent. Phosphorylation of a similar (albeit lower M_r) Mg^{2+}-dependent protein has been described in liver ER (Fleschner et al., 1985).

The transport of $^{45}Ca^{2+}$ showed strong substrate specificity for ATP (Fig. 13). However, the hydrolytic activity was somewhat non-specific for the different nucleotide triphosphate substrates tested (Fig. 19), displaying a difference between the high-affinity Ca^{2+}-ATPase and the Ca^{2+}-transport activities. The substrate specificity of Ca^{2+} transport in our preparations was similar to that reported for the Ca^{2+}-pump of rat pancreatic plasma membranes (Bayerdörffer et al., 1985a) However, it differed from the substrate specificty of the Ca^{2+}

transporter of guinea-pig pancreatic endoplasmic reticulum (Galvan and Lucas, 1987). The substrate specificity of the Ca^{2+}-dependent hydrolytic activity in our preparations generally appeared to be different from the specificities of similar activities found in some other systems studied, such as the liver plasma membrane Ca^{2+}-ATPase (Lotersztajn et al., 1981), or even guinea-pig pancreatic acinar ER (Galvan and Lucas, 1987). Only the liver plasma membrane Ca^{2+}-ATPase enzyme characterized by Lin and coworkers showed a broad substrate specificity similar to that of our preparation (Lin, 1985; Lin and Russell, 1988). This lack of specificity for hydrolytic activity is better illustrated by the 'Mg^{2+}-ATPase' activity (Fig 19b), which compares well with that observed in rat liver plasma membranes (Lin and Russell, 1988). The low hydrolytic activity of the guinea-pig enzyme towards ADP indicates that it is distinct from the plasma membrane ectopic diphosphohydrolase activity previously described in the rat (Ansah et al., 1984) and pig pancreas (LeBel et al., 1980). The function of ecto-ATPases is not known. The pancreatic diphosphohydrolase has been shown to be associated with zymogen granules and may be involved in storage and exocytosis of zymogens (LeBel and Beattie, 1985).

REGULATION OF Ca^{2+}-ATPASE AND Ca^{2+}-TRANSPORT

The effects of potential regulators on the Ca^{2+}-uptake and Ca^{2+}-ATPase activities in guinea-pig pancreatic acinar plasma membrane preparations are summarized in Table VI.

Although C-subunit has been shown to stimulate the erythrocyte and cardiac sarcolemmal Ca^{2+}-pump (Caroni and Carafoli, 1981b; Neyes et al., 1985), there is disagreement as to whether it affects the rate of Ca^{2+} transport or the affinity for Ca^{2+}. Whereas one study reported an increased affinity for Ca^{2+} without an effect on the maximal velocity (Neyes et al., 1985), the other

Table VI. Summary of the effects of different potential regulators on the Ca^{2+}-uptake and Ca^{2+}-ATPase activities of guinea-pig pancreatic acinar plasma membrane preparations. S - stimulation, I - inhibition, NC - no change, nd - not determined.

Regulator	Ca^{2+}-Uptake	Ca^{2+}-ATPase
PROTEIN KINASE A		
C-Subunit	S	NC
HA 1004	NC	NC
C-Subunit + HA 1004	S[a]	nd
CALMODULIN		
CaM	NC	NC
Compound 48/80	I	I[b]
TFP	I	NC
CGS 9343B	NC	NC
PROTEIN KINASE C		
PS	NC	NC
SADG	NC	S
TPA	NC	S
PK-C	NC	S
CGP 41 251	NC	NC

[a]Stimulation compared to control, i.e. no change in C-subunit-stimulated activity
[b]Inhibition at some concentrations; see text

study showed increased maximal velocity with no effect on affinity (Caroni and Carafoli, 1981b). In our experiments, C-subunit stimulated the maximal velocity of Ca^{2+} transport into plasma membrane vesicles more than two-fold without affecting the affinity of the pump for Ca^{2+} (Fig. 20). While these results are in agreement with those of Caroni and Carafoli (1981b), they conflict somewhat with the findings of Neyes et al. (1985). However, they do suggest that the Ca^{2+} efflux from pancreatic acini mediated by the Ca^{2+}-pump may be regulated by protein kinase A. The exact mechanism of this regulation is as yet unknown and requires further investigation. One possibility is a direct phosphorylation of the ATPase as suggested for the sarcolemmal Ca^{2+}-pump (Neyes et al., 1985). The lack of stimulation of Ca^{2+}-ATPase activity by C-subunit demonstrates another difference between this activity and Ca^{2+}-transport activity within our preparations.

HA 1004, a specific inhibitor of protein kinase A, was found to be ineffective in blocking the control and C-subunit-stimulated Ca^{2+} transport and Ca^{2+}-ATPase activities in pancreatic acinar plasma membranes (Table III and Fig. 21). In whole cell experiments, Muallem et al. had noted no effect of HA 1004 on the ability of rat pancreatic acinar cells to 'resist' the increased permeability induced by ionomycin (Muallem et al., 1988b). From these results, they concluded that PKA has no role in the regulation of the pancreatic acinar plasma membrane Ca^{2+}-pump. However, our results with C-subunit disagree with that interpretation. We suggest that HA 1004 may not be a useful inhibitor of the actions of PKA, at least in pancreatic acinar plasma membrane preparations.

Both the Ca^{2+}-pump and Ca^{2+}-dependent ATP hydrolytic activity of guinea-pig pancreatic acinar plasma membranes were insensitive to exogenous CaM (Figs. 10 and 16). This contrasts to the CaM stimulation observed for a

number of other plasma membrane Ca^{2+}-transporting ATPases, such as the enzymes of erythrocytes (Carafoli et al., 1986), bovine sarcolemma (Caroni et al., 1983), toad and pig stomach smooth muscle (Lucchesi et al., 1988) and rat pancreatic acinar cell (Ansah et al., 1984). In a more recent study of rat pancreatic acinar plasma membrane Ca^{2+}-ATPase (Ochs et al., 1988), CaM was shown to have no effect. Furthermore, the plasma membrane Ca^{2+}-ATPases from rat liver (Lin and Fain, 1984; Lotersztajn et al., 1981) and corpus luteum (Verma and Penniston, 1981) have also been reported to be insensitive to CaM. Therefore, it would appear that the universitality of CaM-stimulation of Ca^{2+}-ATPase has not been unequivocally established. Nevertheless, the lack of stimulation of Ca^{2+}-dependent ATP hydrolysis and Ca^{2+} transport activity of our guinea-pig preparations by exogenous CaM may simply reflect the presence of saturating levels of endogenous CaM which may be fully activating the pump *in situ*. Further experiments were undertaken to investigate this possibility.

A concentration-dependent inhibition of $^{45}Ca^{2+}$-uptake by TFP and compound 48/80 (Figs. 22 and 23) suggests the presence of endogenous CaM. However, the complete inhibition of Ca^{2+} transport activity seen in the presence of 1 mM TFP was probably due to non-specific changes in the microenvironment of the enzyme (Seeman, 1977). On the other hand, the two inhibitors failed to produce an inhibition of Ca^{2+}-dependent ATPase activity at different Ca^{2+} concentrations tested (with the exception at 1 μg/ml compound 48/80). The reason for the inhibition with 1 μg/ml compound 48/80 (Fig. 25) is not clear. Compound 48/80 is reported to be much more selective than TFP at inhibiting Ca^{2+}-ATPase activity (Gietzen et al., 1983). However, this would not explain the lack of inhibition at higher concentrations of compound 48/80. Furthermore, we currently have no explanation for the reproducible concentration-dependent stimulation of "Basal" (Mg^{2+}-ATPase) and "Total" (in the presence of both Ca^{2+}

and Mg^{2+}) ATPase activities by compound 48/80 (Fig. 26). It is assumed, however, that this effect is not due to inhibition of endogenous CaM. Whether this effect is somehow related to the histamine releasing properties of compound 48/80 or its recently reported ability to stimulate GTPase activity (Mousli et al., 1990), is not immediately apparent. The dissimilar effects of TFP and compound 48/80 on Ca^{2+}-transport and Ca^{2+}-dependent ATPase activities once again suggest that the two activities are distinct.

The presence of endogenous CaM in our preparations was confirmed in experiments showing stimulation of CaM-sensitive erythrocyte membrane Ca^{2+}-ATPase by the boiled extracts of guinea-pig pancreatic acinar plasma membranes (Fig. 27). The unusually high affinity of Ca^{2+}-uptake for Ca^{2+} in our preparations may also indicate the presence of endogenous CaM; a 50-fold difference in the apparent affinity for Ca^{2+} has been reported for purified sarcolemmal Ca^{2+}-ATPase assayed in the presence and the absence of CaM (Caroni et al., 1983). The pancreas contains one of the highest known concentrations of CaM in mammalian cells (Klee et al., 1980). For example, dog pancreas has been reported to contain 190 mg CaM/kg of tissue (Bartelt et al., 1986). Furthermore, the presence of a multifunctional CaM-PK, presumably responsible for many of the actions of CaM, has been reported in pancreas from rat (Burnham and Williams, 1984; Gorelick et al., 1983) and dog (Bartelt et al., 1986). This protein kinase has been purified from rat pancreatic acinar cells (Cohn et al., 1987). Another kinase, CaM-PK III, having a somewhat different and unique substrate specificity, has also been reported to be present in rat pancreas (Nairn et al., 1985). However, it still remains to be determined whether CaM acts via a CaM-PK or via a direct interaction with the Ca^{2+}-transporting ATPase molecule, as has been observed in the erythrocyte (Carafoli et al., 1986). The suggestion that a 133,000 daltons CaM-binding protein in rat

pancreatic acinar plasma membranes may be the Ca^{2+}-ATPase (Ansah et al., 1984) implies a direct interaction between the two proteins. However, no evidence was provided by these workers to support the proposal that this CaM-binding protein was indeed the Ca^{2+}-ATPase.

Attempts to deplete possible endogenous CaM by use of the EGTA-washing method of Caroni & Carafoli (1981a)) were unsuccessful, indicating that this protein may be tightly bound, as seen in liver plasma membranes (Gazzotti et al., 1985; Gloor and Gazzotti, 1986) and heart sarcolemma (Caroni and Carafoli, 1981a). Further studies using Sephadex G-75 column chromatography appeared promising (Fig. 28), but were abandoned due to low protein recoveries using this procedure.

It has been suggested that PKC may regulate Ca^{2+} extrusion by the plasma membrane Ca^{2+}-transporting ATPase in erythrocytes (Smallwood et al., 1988), cultured vascular smooth muscle cells (Furukawa et al., 1989) and rat pancreatic acinar cells (Muallem et al., 1988b). Studies by Muallem and colleagues used whole cells to determine the effects of H-7 (a protein kinase C inhibitor) and TPA on ionomycin-induced increases in intracellular free Ca^{2+} levels. The changes in free Ca^{2+} were interpreted by those authors as effects of these agents upon PKC to alter the ability of the cell to extrude Ca^{2+} via the Ca^{2+}-pump. To investigate the possible role of PKC in the stimulation of Ca^{2+} efflux from pancreatic acinar cells, we studied the effects of purified rat brain PKC, as well as its activators TPA and SA-DG and an inhibitor CGP 41 251, directly on the $^{45}Ca^{2+}$ transport into plasma membrane vesicles. Purified PKC, the phorbol ester TPA and the diacylglycerol analog SA-DG failed to stimulate Ca^{2+}-uptake (Figs. 30 and 31). These data suggest that PKC may not be involved in the regulation of the plasma membrane Ca^{2+}-pump in guinea-pig pancreatic acinar cells. By contrast, purified PKC stimulated Ca^{2+}-ATPase

activity in the presence of PS at 1 μM free Ca^{2+} (Table V). The potentiation of Ca^{2+}-ATPase activity by TPA and SA-DG in the presence of PS may represent a stimulation of an endogenous PKC. The stimulation by SA-DG and purified PKC noted only at high Ca^{2+} concentrations (Table V) agrees with previous reports that demonstrated an increased V_{max} of Ca^{2+}-transport with PKC without concomittent alteration in K_{Ca} (Furukawa et al., 1989; Smallwood et al., 1988). CGP 41 251, a potent and selective inhibitor of PKC (IC_{50} for PKC = 50 nM, for PKA = 2.4 μM) (Meyer et al., 1989), had no effect on either the Ca^{2+}-transport or the Ca^{2+}-ATPase activity (Tables III and IV). Therefore, our studies provide evidence contrary to the conclusions drawn by Muallem et al. (1988b) who implicate PKC in the regulation of the plasma membrane Ca^{2+}-pump. These results also point to another difference between Ca^{2+}-transport and Ca^{2+}-ATPase activities in guinea-pig pancreatic acinar plasma membranes.

As discussed in the INTRODUCTION, IP_3 and IP_4 have both been suggested to stimulate Ca^{2+} influx into various cell types by a possible action on Ca^{2+} channels in the plasma membrane. To investigate whether these pathways might operate in pancreatic acinar cells, the effects of these two inositol phosphates were studied on Ca^{2+}-transport. If the inositol phosphates were acting on Ca^{2+} channels within pancreatic acinar plasma membranes, $^{45}Ca^{2+}$-uptake into membrane vesicles should be impaired due to the opposing leakage through these channels. We found that neither agent produced a significant effect on Ca^{2+}-uptake (Fig 32), suggesting that IP_3- and/or IP_4- mediated Ca^{2+} releasing pathways may not operate in the isolated guinea-pig pancreatic acinar plasma membrane vesicles.

IS THE HIGH AFFINITY Ca^{2+}-ATPASE THE Ca^{2+}-PUMP?

The similar high affinity of the Ca^{2+}-uptake and the Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase may indicate a possible involvement of this latter activity in Ca^{2+} transport. Furthermore, both activities were insensitive to CaM stimulation and showed an apparent dependency on external Mg^{2+}. However, despite these similarities, the high affinity Ca^{2+}-ATPase and the Ca^{2+} transport activities appear to differ in the guinea-pig pancreatic acinar plasma membrane vesicles: The maximal velocity for ATPase activity was seven-fold higher than that for Ca^{2+}-transport (V_{max} = 6.04 vs. 0.83 nmoles/mg/min, respectively). Whereas Ca^{2+}-transport was highly specific for ATP as substrate, the hydrolytic activity was somewhat non-specific. The two activities also differed with respect to their regulation: While Ca^{2+}-uptake is stimulated by C-subunit, Ca^{2+}-ATPase is not. Although CaM failed to stimulate both activities, CaM antagonists only inhibited Ca^{2+}-transport. Finally, the Ca^{2+}-ATPase activity showed apparent stimulation by both endogenous and exogenous PKC, while Ca^{2+}-transport was unaffected.

The lack of correlation between the Ca^{2+}-transport and the ATPase activities observed in guinea-pig pancreatic acinar plasma membrane preparations suggests that the two activities are probably not related. Similar conclusions were recently drawn by Ochs et al. (1988). Rat liver and corpus luteum have also been suggested to contain two different activities (Birch-Machin and Dawson, 1988; Lin and Russell, 1988; Minami and Penniston, 1987). The suggestion that a high affinity Ca^{2+}-ATPase in liver plasma membranes is a Ca^{2+} transporter (Lotersztajn et al., 1981; Pavoine et al., 1987) is still under dispute (Lin, 1985; Lin and Russell, 1988). This latter controversy may have arisen as a result of the use of different methods of membrane isolation which affect the relative yield of canalicular Vs. sinusoidal plasma

membranes (Birch-Machin and Dawson, 1988). It is suggested that the high affinity Ca^{2+}-ATPase observed in the present studies is not the biochemical expression of the Ca^{2+} pump.

Ca^{2+}-uptake in guinea-pig pancreatic acinar plasma membrane vesicles may be due to an enzyme of low activity whose ATPase activity is not detected in the presence of the higher specific activity Ca^{2+}-dependent ATPase. A similar situation has been observed previously in corpus luteum (Minami and Penniston, 1987) and liver plasma membranes (Birch-Machin and Dawson, 1988). Assuming that the major portion of the high affinity Ca^{2+}-ATPase activity is due to a non-specific ATPase, which is masking the Ca^{2+}-pumping enzyme activity, Kelly and Smith (1987) devised a simple strategy to dissect out the latter activity. Using GTP to block the non-specific ATPase, these workers were able to show the presence of a high affinity Mg^{2+}-dependent Ca^{2+}-ATPase activity with V_{max} and K_{Ca} similar to the Ca^{2+} transporter.

PURIFICATION OF THE Ca^{2+}-PUMP

Because of the heterogeneity of ATPase activity within pancreatic acinar plasma membranes, a complete characterization of the high affinity Ca^{2+}-ATPase which drives transmembrane Ca^{2+} transport must await its isolation to homogeneity and its subsequent reconstitution into defined lipid systems. Accordingly, purification of the Ca^{2+}-transporting ATPase was attempted using CaM-affinity chromatography (Caroni and Carafoli, 1981a; Niggli et al., 1979a) and dye-ligand affinity chromatography (Coll and Murphy, 1984; Scopes, 1987). CaM-affinity chromatography was chosen for two reasons: First, the demonstration of indigenous plasma membrane-bound CaM indicated the possibility of its direct interaction with Ca^{2+}-ATPase; second, the demonstration of a 133,000 daltons CaM-binding protein in ^{125}I-CaM gel-overlay experiments

in rat pancreatic acinar plasma membranes which was suggested to be a Ca^{2+}-ATPase candidate (Ansah et al., 1984). Dye-affinity chromatography was chosen because dye-ligands, including Cibacron Blue 3GA and Reactive Red 120, are known to bind proteins with a dinucleotide-binding fold such as ATPases (Coll and Murphy, 1984; Scopes, 1987). After a number of attempts with both types of affinity chromatography systems, we were unable to purify the Ca^{2+}-ATPase.

STUDIES WITH THE HUMAN PANCREATIC ACINAR PLASMA MEMBRANES

Preliminary studies with human pancreatic acinar plasma membranes demonstrated the presence of a Ca^{2+}-dependent ATPase activity (Fig. 34). Exogenous CaM produced a pronounced stimulation of this activity (Fig. 35), indicating that either these membranes are devoid of endogenous CaM or the enzyme is not fully stimulated by it. The observed lack of inhibition of Ca^{2+}-dependent ATPase by TFP tends to favour the former possibility, i.e. the absence of endogenous CaM from our human pancreatic acinar plasma membrane preparations. This CaM-activated, Ca^{2+}-stimulated ATPase activity may represent the Ca^{2+} extrusion system of human pancreatic acinar cells.

ORIGINAL CONTRIBUTIONS TO THE LITERATURE

1. Demonstrated a highly specific high affinity Ca^{2+}-transporter and a high affinity broad specificity Ca^{2+}-ATPase in the same preparation of guinea-pig pancreatic acinar plasma membrane vesicles.
2. Demonstrated that these two activities are not related.
3. Demonstrated that Ca^{2+}-transport in guinea-pig pancreatic acinar plasma membrane vesicles is stimulated by protein kinase A and endogenous CaM, but not by PKC.

SUMMARY AND CONCLUSIONS

1. These studies demonstrate the presence of an ATP requiring high-affinity Ca^{2+}-pump in guinea-pig pancreatic acinar plasma membranes. This pump may be fully stimulated by endogenous CaM.
2. The unusually high affinity of the Ca^{2+}-pump for Ca^{2+} would indicate that it may play a role in Ca^{2+} extrusion from acinar cells, both at rest and during stimulation.
3. Also present in these preparations was a high-affinity Ca^{2+}-activated, Mg^{2+}-dependent ATPase activity which could only be observed in the presence of high Mg^{2+}.
4. Ca^{2+}-transport was highly specific for ATP as substrate, but the high affinity Ca^{2+}-ATPase was somewhat less specific.
5. Ca^{2+}-transport was stimulated 2-fold by catalytic subunit of protein kinase A, while Ca^{2+}-ATPase was unaffected. CaM antagonists, compound 48/80 and TFP inhibited Ca^{2+}-transport. Purified PKC and its activators, TPA and SA-DG failed to stimulate Ca^{2+}-transport. The results suggest a possible role for CaM and protein kinase A, but not protein kinase C, in the regulation of Ca^{2+} efflux from pancreatic acinar cells.
6. The exact relationship of the high affinity Ca^{2+}-ATPase activity to Ca^{2+}-transport is unclear at present. However, studies to date suggest that the Ca^{2+}-ATPase activity characterized here is not the biochemical expression of the Ca^{2+} pump in these preparations.
7. A number of different strategies failed to yield an acceptable means for purification of the Ca^{2+}-pump protein. The complete elucidation of the characteristics and regulation of the high affinity Ca^{2+}-ATPase activity which drives transmembrane Ca^{2+} transport must await its isolation to homogeneity and its subsequent reconstitution into defined lipid systems.

8. Preliminary studies have demonstrated a CaM-activated, Ca^{2+}-stimulated ATPase activity in human pancreatic acinar plasma membrane preparations.

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