STUDIES ON THE ROLE OF Ca^{2+}
IN THE PANCREATIC ACINAR CELL

By

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ABSTRACT

Calcium ions play an important role in the stimulation of enzyme secretion from the exocrine pancreas. It has been proposed that a secretagogue-induced rise in cytoplasmic free calcium triggers enzyme release from pancreatic acinar cells.

The mechanism by which Ca^{2+} can regulate enzyme secretion was investigated using isolated intact pancreatic acinar cells and plasma membrane-enriched preparations obtained from these cells. The viability of the cells isolated was greater than 95% as evaluated by the exclusion of trypan blue dye. Secretin, pancreozymin and carbachol produced a dose-dependent release of amylase in the acinar cells.

The role of the calcium binding protein, calmodulin in enzyme secretion was studied indirectly by determining the effects of calmodulin antagonists on the secretory process. Trifluoperazine inhibited carbachol-stimulated amylase release (ED_{50} of 10 μM). Chlorpromazine at a concentration of 10 μM inhibited both carbachol- and pancreozymin-stimulated amylase release to a significant degree without affecting secretin stimulated (non-calcium mediated) release. Propranolol did not show this selective inhibition. These studies indicate the possibility that phenothiazines inhibit calcium-mediated amylase release from pancreatic acinar cells by acting on a calmodulin-regulated step in the stimulus-secretion coupling process.

A high ATP hydrolytic activity in the presence of either Mg^{2+} or Ca^{2+} was localized in acinar cell preparations enriched in plasma membranes. Kinetic analysis revealed that the enzyme had a significantly higher affinity ($p < 0.05$) for Ca^{2+} (K_d of 1.73 μM) than Mg^{2+} (K_d of 2.98

μM) but a similar maximal rate of activity. Further studies were carried out in order to determine if this ATPase activity represented one or more enzymes. A comparison of substrate requirements revealed very similar profiles for the Mg^{2+}- and Ca^{2+}-stimulated activities with ATP and GTP being the most effectivefollowed by ADP. The pH curves for both activities were identical. In addition, combinations of saturating concentrations of Mg^{2+} or Ca^{2+} produced the same degree of maximal activity. These data tend to indicate that the ATPase activity may be due to one enzyme with activation sites for both ions. Arrhenius plots though, revealed a difference in the transition temperatures. In addition, when SDS polyacrylamide gel electrophoresis was used to identify the phosphoprotein intermediates of the ATPase, one phosphoprotein intermediate (approx M.W. of 115,000) was observed in the presence of Ca^{2+} alone (endogenous Mg^{2+} =1.5-2 μM) but upon the addition of both Ca^{2+} and Mg^{2+}, a second phosphoprotein intermediate was revealed (M.W. 130,000). Both of these phosphoproteins were hydroxylamine-sensitive, indicating that they were acyl phosphates. The presence of two acyl phosphoproteins would indicate possible separate intermediate reaction systems for the Mg^{2+} and Ca^{2+} stimulated ATPase activity.

Calmodulin is known to regulate a number of enzyme systems including Ca^{2+}-transport ATPases. The effect of exogenous calmodulin on the Ca^{2+}-stimulated component of this ATPase activity was therefore investigated. In the presence of endogenous Mg^{2+}, significant stimulation by calmodulin of Ca^{2+}-ATPase activity was observed. This effect was dose-dependent with a K_d for calmodulin of approximately 0.7 μM. Calmodulin increased the Ca^{2+}-sensitivity of this enzyme

system; Mg^{2+} appeared to be required for this effect. This calmodulin stimulation was inhibited by trifluoperazine (ED_{50} of 30 μM), chlorpromazine (ED_{50} of 55 μM) and compound 48/80. Using an ^{125}I-labeled calmodulin gel overlay technique, it was shown that calmodulin binds in a Ca^{2+}-dependent fashion to 133,000 and 230,000 dalton proteins present in the plasma membrane-enriched fraction. Further studies revealed that under conditions that favour Ca^{2+}-dependent kinase activity, calmodulin enhanced the phosphorylation of a 30,000 and a 19,000 dalton protein. The Ca^{2+}-ATPase was also stimulated by acidic phospholipids.

Ca^{2+}-flux measurements in plasma membrane vesicles indicated the presence of two processes: there were significantly lower levels of ^{45}Ca associated with vesicles 'loaded' in the presence of ATP than those 'loaded' in the absence of ATP indicating an energy-dependent efflux system. As well, a very rapid ATP-dependent Ca^{2+}-uptake system, that was Mg^{2+}-dependent was observed in sealed vesicles.

In order to relate the (Mg^{2+} + Ca^{2+})-ATPase activity and the Ca^{2+} fluxes observed to a possible functional role in the acinar cell, the orientation of the catalytic site of the enzyme was determined. The degree of ATPase activity observed when intact viable acinar cells were incubated with ATP and either Mg^{2+} or Ca^{2+} was similar to that observed in broken membranes. This provides evidence that this ATPase activity may be externally-oriented. The role of this system in acinar cell function remains to be elucidated.

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ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
Ap5A	adenyl 3',5'-adenosine
GTP	guanosine triphosphate
P_i	inorganic phosphate
cyclic AMP	cyclic adenosine 5'-monophosphate
cyclic GMP	cyclic guanosine 5'-monophosphate
E	enzyme
EP	phosphorylated enzyme intermediate
ATPase	adenosine triphosphatase
$(Ca^{2+} + Mg^{2+})$-ATPase	Mg^{2+} dependent calcium stimulated ATPase
EDTA	ethylenediamine tetraacetate, disodium salt
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
SEM	standard error of the mean
Tris	tris(Hydroxymethyl) aminomethane
TCA	trichloroacetic acid
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
TFP	trifluoperazine
CPZ	chlorpromazine
NADH	nicotinamide adenine dinucleotide (reduced)
$K_{Ca^{2+}}$	dissociation constant of the enzyme for Ca^{2+}

g	Gravitational Constant
K_{diss}	dissociation constant
Vmax	maximum velocity
m	milli
μ	micro
M	molar
M_r	molecular weight
mg	milligram
sec	second
min	minute
ml	milliliter
μl	microliter
mM	millimolar concentration
μM	micromolar concentration
nmoles	nanomoles
v/v	unit of volume per unit of volume
w/v	unit of weight per unit of volume

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INTRODUCTION

Cystic fibrosis (CF) is an inherited metabolic disease which is characterized by a universal dysfunction in the exocrine glands of affected individuals. The basic molecular defect and the pathogenetic mechanisms of the disease is still unknown. It has long been recognized that mucus secretions from CF patients have abnormal physicochemical properties. They precipitate and obstruct organ passages, giving rise to chronic pulmonary disease, pancreatic insufficiency, hepatic cirrhosis, intestinal obstruction and other complications (di Sant'Agnese and Davis 1976). Large decreases in the amount of pancreatic juice produced as well as elevations in the protein and calcium content of the juice have been reported (Hadorn et al 1968; Johansen et al 1968). The abnormalities of exocrine secretions could either be related to the synthesis and secretion of macromolecules or the result of their compositional modifications in the duct systems.

Recently, Mangos and Donnelly (1981) have demonstrated that isolated parotid acinar cells from patients with CF are larger than cells from controls and have higher concentrations of amylase, calcium and sodium and lower concentrations of potassium which is suggestive of a defect in the acinar cell physiology of these patients. However, the cellular control mechanisms responsible for electrolyte, water and macromolecular secretion in exocrine tissue are not fully understood. Although the pancreas is one of the glands most profoundly affected by the disease, very little information is presently available regarding the molecular events involved in the secretion of proteins.

LITERATURE REVIEW

1. Structure and Function of the Exocrine Pancreas

The pancreas comprises exocrine and endocrine tissue. The exocrine tissue, in addition to secreting digestive hydrolases, also secretes a fluid containing electrolytes. Together these form pancreatic juice which enters the duodenum via the pancreatic duct (Case 1978). The endocrine tissue, which accounts for only 4% of the gland in the rat (Kempen et al 1977) secretes insulin, glucagon, somatostatin and what is now being referred to as pancreatic polypeptide (Kimmel et al 1975).

The functional unit of the exocrine pancreas consists of pyramidal-shaped acinar cells grouped around a narrow lumen to form an acinus. The terminal cells of the acinar lumen are called centro-acinar cells. The major function of the pancreatic acinar cells which comprise 77% of the pancreatic cell mass in the rat (Kempen et al 1977) is to synthesize and secrete a variety of digestive enzymes while the centro-acinar cells secrete a bicarbonate-rich fluid important in neutralizing gastric acid in the intestine.

A. Secretory Products

Using two-dimensional gel electrophoresis, which separates proteins in the first dimension according to their isoelectric point and in the second dimension according to their molecular weight, Scheele (1975) showed that the acinar cell of the exocrine pancreas secretes a mixture of approximately 20 enzymes and zymogens. These include proteases

(trypsinogen and chymotrypsinogen), amylase, lipase and ribonucleases. Chymotrypsinogen, proelastase, procarboxy-peptidase A, and lipase appeared in multiple forms.

B. The Secretory Pathway

Pancreatic enzymes, like all proteins, are synthesized on polysomes (aggregations of ribosomes) joined to the rough endoplasmic reticulum (RER). From their site of synthesis, they move into the so-called transitional elements. What appears to occur next is that the transitional elements bud off the RER as small transporting vesicles that carry their load of secretory proteins to the Golgi complex. The secretory proteins undergo extensive modifications during their residence in the Golgi complex. Following fusion of the transporting vesicles with the Golgi stacks, saccules nearest the concave side gradually fill with secretory material, become rounded up, and eventually bud off as an immature storage granule. Gradually, the concentration of proteins in the "condensing vacuole" increases. The eventual result is the zymogen granule packed with exportable proteins. Under appropriate external stimuli, these granules fuse with the apical plasmalemma, ejecting their contents into their acinar lumen by the process of exocytosis (Case 1978).

Experimental evidence on the route and time course of intracellular transport of secretory proteins was obtained mainly from the classic electron microscopic and autoradiographic studies of Jamieson and Palade (1967a and b; 1971a and b; 1977). Using guinea pig pancreatic slices

and an in vitro 'pulse-chase' technique, they determined that proteins

pulse-labeled with [^{3}H]-leucine first appeared in the area of the cell

occupied by the RER. After 17 min of 'chase' with a complete mixture of

non-radioactive amino acids, maximum labeling was associated with the

Golgi complex. After 37 min of 'chase' autoradiographic grains were

observed on the side of the Golgi complex facing the apical membrane and

many grains were associated with condensing vacuoles. After 57 min of

'chase', most of the grains were observed in the apical portion of the

acinar cell in association with mature zymogen granules. The route and

time course of movement of pulse-labeled proteins were found to be the

same in unstimulated pancreatic slices or in slices that had received a

prior three-hour stimulation with carbachol (Jamieson and Palade

1971b). Bieger et al (1976) however found that continuous stimulation

(6-24h) of the in vitro rat pancreas with caerulein accelerated to a

considerable extent the rate of movement of pulse-labeled proteins along

this pathway.

Cell fractionation techniques, in which the rough microsomes, smooth

microsomes, zymogen granules and post-microsomal supernatants were

separated by differential rate sedimentation following tissue

homogenization, confirmed the autoradiographic findings (Jamieson and

Palade 1967a). It also determined that the kinetics of movement of

pulse-labeled proteins from one membrane-enclosed compartment to another

was neither sharp nor unambiguous. This was additionally complicated by

the appearance of significant quantities (5-20%) of pulse-labeled

protein in the postmicrosomal supernatant fraction at all time points

studied. These findings have given rise to another school of thought

which suggests that under physiological conditions secretory proteins pass through the cytoplasmic space during their intracellular transport (Rothman 1980). Recent findings of Scheele and co-workers (1978) have indicated that the ambiguity seen in early studies was secondary to leakage and adsorption artifacts associated with tissue homogenization and cell fractionation. The widely held view is that:

a. at all times, secretory proteins are segregated within membrane-bound compartments and therefore excluded from the cytosolic space;

b. transport of secretory proteins is vectorial and irreversible proceeding from the basilar portion of the cell to the apical portion through a series of interconnectable membrane-enclosed compartments; and

c. transport is time-dependent requiring, under normal conditions, approximately 30 min to position secretory proteins for discharge into the extracellular space (Jamieson and Palade 1971b; Scheele and Palade 1975).

Studies have been conducted to determine whether the enzymes and zymogens of the exocrine pancreas are synthesized, stored and secreted as a group by a single secretory pathway (parallel discharge) or individually by a multiplicity of secretory pathways (non parallel discharge). The available evidence seems to favour a single secretory pathway: Immunocytochemical localization studies of Kraehenbuhl et al (1977) indicated that each of five monospecific antibodies directed toward individual bovine pancreatic proteins (trypsinogen,

chymotrypsinogen A, carboxypeptidase A, ribonuclease, and
deoxyribonuclease) reacted with the content of all zymogen granules in
all bovine pancreatic acinar cells. Although these findings do not
provide quantitative information, they indicate that each zymogen
granule in each acinar cell contains a mixture of the five secretory
proteins tested. Studying guinea pig pancreatic lobules, Scheele and
Palade (1975) used optimal concentrations of carbachol or caerulein and
followed the discharge of three enzymes, amylase, lipase and
ribonuclease and four zymogens, trypsinogen, chymotrypsinogen and
procarboxypeptidases A and B into the incubation medium at varying time
intervals up to 2h after stimulation. They (Scheele and Palade 1975)
found that each of the enzymes and zymogens measured were discharged in
constant proportion at each time point. Furthermore, the proportions of
these enzymes and zymogens found in the tissue at the end of the
experiment were identical to those discharged into the medium. Parallel
discharge of enzymes upon stimulation have been confirmed by other
workers (Glazer and Steer 1977; Rothman and Wilking 1978). However,
while Scheele and Palade (1975) and Steer and co-workers (Steer and
Glazer 1976; Steer and Manabe 1979) in their in vitro studies found that
the proportion of enzymes and zymogens were unchanged when stimulated
discharge was compared to basal discharge, in vivo studies on the rat
(Dagorn 1978), pig (Beaudoin 1983) and human subjects (Rinderknecht et
al 1978) indicated that the proportion was changed. Though these
nonparallel patterns of secretion could be viewed as supporting the
existence of an alternative secretory pathway which involves the

movement of secretory proteins through the cytoplasmic space (Rothman 1980) it is also possible that one population of acinar cells might be responsible primarily for basal discharge and another for stimulated discharge (Scheele 1980).

C. Secretagogues of Enzyme Secretion

The physiological stimulants of enzyme secretion from pancreatic acini are the neurotransmitter acetylcholine (ACh) and the oligopeptide cholecystokinin-pancreozymin (CCK). With recent progress in the development of in vitro preparations such as slices of pancreas, intact acini and dispersed acinar cells, many different agents have been found to be capable of increasing pancreatic enzyme secretion. The generally, recognized groups are:

a. Muscarinic Cholinergic Agents

Acetylcholine and other muscarinic cholinergic agents cause mobilization of cellular calcium as well as stimulation of enzyme secretion in pancreatic acinar cells (Gardner 1979). These actions are blocked by atropine (Gardner 1979). Using tritiated quinuclidinyl benzilate ([^{3}H]QNB), Larose et al (1979) and Ng et al (1979) have demonstrated the presence of muscarinic cholinergic receptors in pancreatic broken cell preparations. However, the corresponding agonist-induced enzyme secretion could not be measured in these preparations (Larose et al 1979; Ng et al 1979). Recently, binding studies with [^{3}H]QNB performed on rat pancreatic dispersed acini with

concomitant secretory studies have established that the muscarinic receptor is coupled to enzyme secretion (Larose et al 1981). The receptor population consisted of high and low affinity sites with a K_d of 65 pM and approximately 2600 binding sites (Larose et al 1981).

b. Cholecystokinin-Gastrin-Caerulein Peptides

Cholecystokinin-pancreozymin is a peptide that was originally isolated and purified from hog upper small intestine and found to contain 33 amino acid residues (Mutt and Jorpes 1968). The two other naturally occurring peptides that are structurally similar to CCK are gastrin and caerulein. Like CCK and ACh (Christophe et al 1976) they stimulate enzyme secretion, increase cellular guanosine 3',5'-cyclic monophosphate (cyclic GMP) and cause the release of cellular calcium from pancreatic fragments and dispersed acinar cells (Deschodt-Lanckman et al 1976; May et al 1978). Gastrin occurs naturally in several different chemical forms and is similar to CCK in its carboxyl(C)-terminal amino acid sequence (Gregory and Tracy 1964). Caerulein is a decapeptide originally isolated from the skin of the Australian hylid frog Hyla caerulea (Anastasi 1968) and subsequently found to share seven of its eight C-terminal amino acids with the C-terminal octapeptide of CCK. The intrinsic biologic activity of CCK resides in the C-terminal portion of the molecule. The smallest active fragment is the C-terminal tetrapeptide (CCK4). All the active peptides have the same efficacy but varying potencies (Jensen and Gardner 1981). The most potent are CCK8 and caerulein (10 times more potent than

native CCK). Studies with ^{125}I-labeled CCK33 have revealed
high-affinity binding sites on pancreatic acinar cells (Jensen et al
1980; Sankaran et al 1980). From the studies of Jensen et al (1980), it
appears that there may be approximately 9000 binding sites per acinar
cell with a K_d of about 2nM for CCK33 and 0.3 nM for caerulein.
Butyryl derivatives of cyclic GMP selectively and competitively inhibit
various physiological responses to stimulation with CCK peptides (Peikin
et al 1979; Philpott and Petersen 1979). These cyclic GMP derivatives
also selectively and competitively block the binding of ^{125}I-labeled
CCK33 to the acinar cells (Jensen et al 1980). Recently, it has been
suggested that the interaction between dibutyryl cyclic GMP and CCK,
rather than receptor blockade, may be the mechanism for inhibition of
CCK activity (Miller et al 1983). However, the lack of certain control
experiments make this interpretation inconclusive. Therefore the
current view remains that dibutyryl cyclic GMP acts as an antagonist of
CCK by interacting with the receptor (Gardner 1983). It has been shown
that proglumide and benzotript, compounds that are structurally very
different from cyclic nucleotides, specifically and competitively
antagonize the binding as well as the actions of the CCK peptides (Hahne
et al 1981).

c. Bombesin-like Peptides

Another group of naturally occurring peptides that can stimulate
pancreatic enzyme secretion by causing release of cellular calcium
comprises bombesin, alytesin, ranatensin and litorin. These peptides,
like caerulein, were originally isolated from the skins of various frogs

and were named after the particular class of frog from which they were
isolated (Erspamer et al 1972; Erspamer and Melchiorri 1973; Erspamer
and Melchiorri 1975). As in the case with CCK, the intrinsic biological
activity of bombesin and structurally related peptides is a property of
the C-terminal portion of the molecule; the C-terminal nonapeptide of
bombesin has the same potency and efficacy as the native
tetradeca-peptide (Deschodt-Lanckman et al 1976; Erspamer and Melchiorri
1973; Erspamer and Melchiorri 1975; Jensen et al 1978; Rivier and Brown
1978). Since bombesin is able to release gastrin and CCK from the gut
(Konturek et al 1976) it was originally thought that bombesin only
activated pancreatic acinar cells indirectly via CCK-gastrin peptides.
Deschodt-Lanckman et al (1976) demonstrated that bombesin evoked
secretion and calcium-flux changes in isolated fragments of rat pancreas
and suggested that bombesin acted directly on the acinar cells. Other
workers (Jensen et al 1978; Iwatsuki and Petersen 1978; Philpott and
Petersen 1979; Petersen and Philpott 1979) have presented clear and
direct evidence showing coexistence of separate CCK and bombesin
activation sites on the same acinar units. Binding studies with a
radiolabeled analogue of bombesin [^{125}I] Tyr^4-bombesin have revealed
high affinity binding sites in guinea pig pancreatic acini. There are
about 5000 bombesin receptors per acinar cell with a dissociation
constant for bombesin of 4 nM (Jensen et al 1978). While direct
bombesin actions on acinar cells have been observed in the mouse, rat
and guinea pig (Deschodt-Lanckman et al 1976; Iwatsuki and Petersen
1978; Petersen and Philopott 1979; Jensen et al 1978) it appears that

dog acinar cells do not respond to bombesin (Bommelaer et al 1980).

d. **Substance P-like Peptides**

Little work has been done on the pancreatic acinar actions of these peptides. In the guinea pig, high affinity binding sites have been demonstrated using [^{125}I] physalaemin (Jensen and Gardner 1981). It appears that there may be about 500 binding sites per cell with a dissociation constant of about 2 nM for physalaemin and 5 nM for substance P (Jensen and Gardner 1981). These peptides appear to act like ACh, CCK and bombesin in evoking calcium release and an increase in cyclic GMP, but the maximal effects are much smaller than those evoked by the other types of secretagogues (Jensen and Gardner 1981).

e. **Secretin and Vasoactive Intestinal Polypeptide**

Secretin and Vasoactive Intestinal Polypeptide (VIP) have a similar spectrum of biological activities that reflects the similarities in their amino acid sequences (Schulz and Stolze 1980). In contrast to the peptides that increase calcium release from acinar cells and have their intrinsic biological activities in the C-terminal portion of the molecule, the intrinsic biological activity of secretin and VIP resides in the N-terminal portion of the molecule (Christophe et al 1976; Robberecht et al 1976). Binding studies (Christophe et al 1976; Robberecht et al 1976; Gardner et al 1979) have demonstrated that acinar cells possess two classes of receptors, each of which interact with VIP and secretin and each causing an increase in adenosine 3',5'-cyclic

monophosphate (cyclic AMP). There is a high affinity VIP receptor (K_d= 0.7 nM) which has a low affinity for the hormone secretin (K_d = 7 nM) and a high affinity secretin receptor (K_d = 0.3 nM) with a relatively low affinity for VIP (K_d = 80 nM). There are approximately 9000 high affinity VIP receptors per acinar cell and about 135,000 high affinity secretin receptors (Gardner et al 1979). Although interaction with both classes of receptors can cause an increase in cyclic AMP, it is only the increase in cyclic AMP evoked by occupation of the VIP-preferring receptors that mediates enzyme secretion. It must be noted that there is a species specificity with regard to the ability of VIP to stimulate enzyme release. VIP evokes enzyme secretion in guinea pig and rat, but not in dog, cat or mouse (Robberecht et al 1977). The possible explanation is that acinar cells from dog, cat and mouse pancreas may possess secretin-preferring receptors that mediate the increase in cyclic AMP but may lack the VIP-preferring receptors that mediate the stimulation of enzyme secretion. One finding in support of this possibility is that studies of ^{125}I-secretin binding to partially purified plasma membranes from cat pancreas showed a single class of binding sites that had a high affinity for secretin and a low affinity for VIP (Milutinovic et al 1976).

Secretin and VIP control the secretion of bicarbonate-rich fluid from pancreatic duct cells by increasing cellular cyclic AMP concentration (Case et al 1980). However, there is no information available with regard to the mechanism by which cyclic AMP turns on the ion-fluid transport machinery nor the precise ion membrane transport mechanisms (Scratcherd et al 1981).

f. Cholera Toxin

In acinar cells from guinea pig and rat pancreas, cholera toxin increases enzyme secretion by virtue of its ability to activate adenylate cyclase and increase cellular cyclic AMP (Gardner and Rottam 1979; Singh 1982). The "B subunit" of cholera toxin, also referred to as choleragenoid can also bind to the cholera toxin receptor and thereby function as a competitive antagonist. The pancreatic acinar cell is estimated to have approximately 21,000 receptors for cholera toxin with a K_d of 1 nM (Gardner and Rottam 1979).

g. Insulin

The finding of high- and a low-affinity binding sites using biologically active ^{125}I-insulin suggests that insulin may directly regulate specific functions in the exocrine pancreas (Korc et al 1978). Studies employing either intact, perfused or pancreatic fragments have indicated that insulin may regulate glucose oxidation (Danielsson and Sehlin 1974) and amylase synthesis and secretion (Soling and Unger 1972; Kanno and Saito 1976). The relationship of insulin binding to stimulus-secretion coupling is not clear. It has been suggested that insulin may not directly stimulate amylase secretion but rather potentiate the effects of hormonal secretagogues (Williams et al 1981).

2. The Role of Calcium and Cyclic Nucleotides in Enzyme Secretion

Pancreatic acinar cells are connected by tight junctions. While the primary function of these "fused" plasma membranes is to prevent

leakage of pancreatic secretions into the intracellular spaces, they also block off the apical region of the cells from the circulation, the means by which the hormones reach their target organs. Thus structural and functional polarization exists, with secretagogue-receptor interaction at the basal membrane and enzyme release at the apical membrane. An intracellular mediator (second messenger) for secretion is therefore required.

A. Calcium

A number of studies in which calcium removal, either alone or with addition of a chelator such as ethyleneglycol-bis(β-aminoethylether)--N,N'tetraacetic acid (EGTA) carried out using incubated pancreatic fragments or perfused pancreas, have emphasized a calcium requirement for amylase secretion (Hokin 1966; Robberecht and Christophe 1971; Benz et al 1972; Heisler et al 1972; 1972; Argent et al 1973). However in contrast to studies on nerve terminals (Katz and Miledi 1967) and posterior pituitary (Douglas and Poisner 1964) in which a dependence on extracellular calcium of secretagogue-induced secretion was clearly established, in the pancreas an immediate blockade of stimulated secretion in the absence of extracellular calcium could not be shown (Williams and Chandler 1975). Williams and Chandler (1975) observed only a 50% depression of bethanechol-stimulated secretion from mouse pancreatic fragments after 30-90 min of preincubation in a Ca^{2+}-free medium in the absence of calcium chelator. In contrast, Hokin (1966) found a complete loss of the ACh response in pigeon pancreatic slices

"presoaked" in a Ca^{2+}-free saline solution containing EDTA and further incubated for 40 min in a Krebs-Ringer bicarbonate medium without calcium.

The significance of these observations is difficult to assess since lack of immediate effects of calcium omission might result from insufficient calcium removal. On the other hand, complete and prolonged removal of extracellular calcium might eventually decrease intracellular calcium or provoke nonspecific damage that might be responsible for the missing response. A study on superfused mouse pancreatic fragments (Petersen and Ueda 1976) showed that amylase secretion 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. However, in a Ca^{2+}-free solution containing EGTA, ACh stimulation, normally causing sustained enzyme secretion, resulted in only a short burst of secretion. It appears that amylase release is independent during the first minutes of secretion but at later times dependent on extracellular calcium. That this may be the case comes from studies in which the effect of caerulein on amylase release from isolated pancreatic acini was examined in medium containing normal calcium and in Ca^{2+}-free medium containing EGTA (Williams 1980). It was observed that caerulein-stimulated amylase release was similar in the two media for the first 10 min, but thereafter the ability of caerulein to stimulate release was lost in the acini suspended in Ca^{2+}-free medium.

Understanding how enzyme release can be controlled by intracellular and/or extracellular calcium required the use of radioisotopes to

monitor calcium fluxes. Initial observations were controversial. In

pancreatic fragments and superfused intact tissue, while ^{45}Ca influx

after addition of secretagogues could be observed by Heisler and Grondin

(1973) using the "lanthanum method", other investigators observed

increased ^{45}Ca efflux from ^{45}Ca preloaded tissue and were not able to

detect any effect on ^{45}Ca influx (Case and Clausen 1973; Matthews et al

1973; Chandler and Williams 1974; Deschodt-Lanckman et al 1976).

Similarly, using enzymatically dissociated dispersed acinar cells, an

increased ^{45}Ca influx was observed by Kondo and Schulz (1976a and b),

while ^{45}Ca efflux from preloaded cells was observed in other studies

(Gardner et al 1975; Christophe et al 1976). The diversity of results ·

could be attributed to whether or not tracer Ca^{2+} exchange was measured

at steady-state and therefore whether the movement of ^{45}Ca was an

indication of net Ca^{2+} flux. From the data obtained by unidirectional

^{45}Ca flux measurements at steady-state conditions (Kondo and Schulz

1976a and b) as well as by measurements of "Ca^{2+} net flux" (Gardner et

al 1975; Renckens et al 1978; Stolze and Schulz 1980), it appears that

calcium fluxes during secretagogue-stimulated enzyme secretion are

biphasic. There is an initial Ca^{2+}-extrusion from the cell due to

release of Ca^{2+} from intracellular stores followed by a reuptake phase

into a compartment different from where calcium has been released.

What remained to be determined were the site(s) of the

secretagogue-evoked calcium release and the biochemical mechanism that

could mediate the calcium transport processes.

Various approaches have been used to determine the site(s) of the

secretagogue-evoked calcium release. Subcellular fractionation carried out on cells or tissues that have been loaded with ^{45}Ca suggested that calcium was released from mitochondria (Clement and Meldolesi 1975a and b), from a microsomal fraction consisting of endoplasmic reticulum, elements of the Golgi complex and perhaps also the plasma membrane (Dormer and Williams 1981). Changes in membrane bound calcium were monitored by Chandler and Williams (1978a and b) using the fluorescent probe chlorotetracycline. It was observed that mitochondrial metabolic inhibitors mimicked the effect of secretagogues on the chlorotetracycline signal. These authors concluded that calcium was released from either the mitochondria or by an organelle requiring ATP produced by mitochondrion. Another approach, using agents with specific sites of action has been employed. Lanthanum at a concentration of 1 mM abolished ^{45}Ca release in response to cholinergic stimulation (Wakasugi et al 1981). Lanthanum was found bound to the plasma membrane, but not inside the cells. Wakasugi et al (1981) therefore concluded that calcium was released from the plasma membrane. The possibility though that lanthanum could be acting on the plasma membrane to interfere with the generation of a signal that controls a trigger calcium pool located elsewhere was not ruled out.

In spite of accumulating evidence suggesting that stimulation of enzyme secretion is triggered by an increase in cytosolic calcium (Schulz 1980; Williams 1980; O'Doherty and Stark 1982; Dormer 1983) the transport mechanism(s) required to critically control the intracellular free calcium concentration is not fully worked out. So far,

electrophysiological studies have failed to identify a calcium component in the ACh-evoked membrane potential and conductance change although sustained ACh-evoked acinar membrane depolarization is acutely dependent on extracellular calcium (Petersen and Maruyama 1983). Rather, the increase in intracellular calcium appears to open up the ionic pathways permeable to sodium, chloride and potassium (Maruyama and Petersen 1982). At present, biochemical studies are being directed towards identifying active calcium transport processes in various cellular organelles. An ATP-dependent Ca^{2+}-transport in pancreatic microsomes, probably consisting of vesicles from both plasma membranes and endoplasmic reticulum has been reported (Argent et al 1975; Lucas et al 1978; Ponnappa et al 1981). The active Ca^{2+}-transport was not inhibited by mitochondrial inhibitors such as antimycin A, azide or oligomycin and was associated with a Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase activity (Ponnappa et al 1981). In acinar cells treated with saponin (Wakasugi et al 1982) or digitonin (Lucas et al 1983) to make the plasma membrane permeable, both mitochondrial and non-mitochondrial Ca^{2+}-uptake systems were identified. A Ca^{2+}-ATPase activity has been described in plasma membrane enriched fractions prepared from whole tissue homogenates (Milutinovic et al 1977). It has been shown by other workers (Forget and Heisler 1976; Lambert and Christophe 1978; Le Bel et al 1980; Martin and Senior 1980) using similar preparations that the ATPase activity is stimulated almost equally by Ca^{2+} or Mg^{2+}. Since the cellular origin of the ATPase activity is not certain its role in the regulation of intracellular calcium in the pancreatic acinar cell still remains obscure.

Another transport mechanism that may play a role in the regulation of intracellular calcium is the Na^+/Ca^{2+} countertransport system. This system uses the electrochemical Na^+ gradient across the plasma membrane as a source of energy and has been well characterized in nerve axons and cardiac muscle (DiPolo and Beaugé 1980; Mulins 1979). Using isolated pancreatic plasma membrane vesicles loaded with ^{22}Na, Schulz and Heil (1979) have shown that an increase in internal Ca^{2+} increased the Na^+ permeability whereas an increase in the external Ca^{2+} decreased Na^+ permeability. This appears to be an important system since it is known from electrophysiological studies that one major effect of cholinergic stimulation is to increase membrane sodium permeability (Maruyama and Petersen 1982).

B. Cyclic GMP

Pancreatic secretagogues that cause release of cellular calcium also increase cellular cyclic GMP (Albano et al 1976; Christophe et al 1976; Deschodt-Lanckman et al 1976; May et al 1978). There is a close correlation between the dose-response curve for the action of a secretagogue on cellular cyclic GMP and that for its action on calcium release (Christophe et al 1976). Three findings suggest that secretagogues increase cellular cyclic GMP by causing mobilization of cellular calcium: i) Calcium can stimulate guanylate cyclase activity in broken cell preparations (Christophe et al 1980). Both particulate and soluble forms of the enzyme have been identified in the pancreas (Christophe et al 1980). Calcium and Mg^{2+} can substitute for Mn^{2+} at

the metal activator site on the soluble form. The particulate form is stimulated by mono and polyunsaturated fatty acids (Christophe et al 1980). ii) The calcium ionophore, A23187 increases cellular cyclic GMP in dispersed acinar cells (Christophe et al 1976). and iii) Exogenous derivatives of cyclic GMP do not cause calcium efflux from in vitro preparations of pancreas (Christophe et al 1976).

The stimulant-evoked increase in cyclic GMP concentration is transient, reaching maximum levels after 1 to 2 min (Christophe et al 1980). The transient elevation of the cyclic GMP concentration despite continued agonist exposure may result from a sequential stimulation of guanylate cyclase and a Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. Indeed, three different forms of cyclic nucleotide phosphodiesterase have been found in the pancreas (Terai et al 1976; Vandermeers et al 1977).

Vandermeers et al (1977) have shown that in the presence of cyclic GMP as substrate the activity of one of these phosphodiesterases (P3) is specifically stimulated by calmodulin. There are two forms of the calmodulin-dependent cyclic nucleotide phosphodiesterase from rat pancreas (M_r 175,000 and 116,000) that exhibit higher affinity for cyclic GMP than cyclic AMP (Vandermeers et al 1983).

The role of cyclic GMP as a potential mediator of the action of secretagogues on pancreatic enzyme secretion is still unclear. Scheele and Haymovits (1979) observed that incubation of guinea pig pancreatic lobules in calcium-free medium containing 1mM EGTA resulted in a severe reduction in carbachol-stimulated enzyme secretion, whereas the tissue

response to cyclic GMP remained largely intact. It was also shown that depolarization of the acinar cell membrane by elevated KCl concentrations, in the presence of atropine, caused enzyme release without elevation of cyclic GMP levels (Scheele and Haymovits 1980). Furthermore, Gunther and Jamieson (1979) showed that nitrosourea compounds elevated cyclic GMP levels dramatically without causing secretion. On the other hand, a phorbol ester, 12-0-tetradecanoyl phorbol-13-acetate, caused marked enzyme secretion with no elevation of cyclic GMP levels (Gunther and Jamieson 1979). There are some reports of a slight stimulatory effect of exogenous derivatives of cyclic GMP on acinar enzyme secretion (Albano et al 1976; Haymovits and Scheele 1976; Gardner and Jackson 1977). However, this observation could not be substantiated by others (Heisler and Grondin 1975; Ueda et al 1980).

C. Cyclic AMP

The increase in amylase secretion elicited by secretin or VIP is accompanied by an increase in cellular cyclic AMP levels (Christophe et al 1976; Robberecht et al 1976; Gardner et al 1979) with no change in calcium flux or cellular cyclic GMP (Gardner et al 1975; Christophe et al 1976). Inhibitors of cyclic nucleotide phosphodiesterase are known to augment the action of secretin or VIP on cellular cyclic AMP levels as well as on enzyme secretion (Deschodt-Lanckman et al 1975; Gardner and Jackson 1977) and both peptides can activate adenylate cyclase in membrane preparations from pancreatic acinar cells (Rutten et al 1972; Svoboda et al 1976; Long and Gardner 1977). Furthermore, derivatives of

cyclic AMP can increase enzyme secretion from the pancreas in vitro (Haymovits and Scheele 1976; Gardner and Jackson 1977); The increase in enzyme secretion produced by secretin or VIP plus exogenous derivatives of cyclic AMP is the same as that obtained with secretin or VIP alone (Gardner and Jackson 1977).

The finding that CCK could increase cyclic AMP concentrations in the pancreas (Deschodt-Lanckman et al 1975) together with the observation that CCK as well as C-terminal fragments and analogs could activate adenylate cyclase in broken cell preparations (Rutten et al 1972; Svoboda et al 1976; Long and Gardner 1977) suggested that cyclic AMP mediated the action of CCK. However, since the concentrations of CCK that increase calcium efflux, cyclic GMP and amylase release do not increase cellular cyclic AMP in intact acinar cells (Long and Gardner 1977), these findings may reflect contamination of the CCK with small amounts of secretin- or VIP-like material.

The mechanism of adenylate cyclase activation in the pancreas is similar to that found in other systems (Cassel and Selinger 1978): It has been shown that GTP and particularly its analog guanylyl imidodiphosphate [Gpp(NH)p], stimulate pancreatic adenylate cyclase activity (DePont et al 1977; Svoboda et al 1978). The stimulatory effect of the GTP analog was higher than that of secretin, CCK8 or fluoride, an ubiquitous activator of membrane-bound adenylate cyclases in animal cells (Perkins 1973).

It was recently shown that electrical stimulation of non-cholinergic, non-adrenergic nerves in isolated guinea-pig pancreatic

segments evokes amylase secretion which is preceded and accompanied by marked and complex changes in cellular cyclic nucleotide cancentrations (Pearson et al 1981). The pattern of cyclic AMP and cyclic GMP concentration changes could be mimicked by exogenous VIP, but not by caerulein. The phophodiesterase inhibitor theophylline had a potentiating effect on both the nerve-stimulation- and VIP-evoked secretory response as well as the increase in cyclic AMP concentration (Pearson et al 1981).

D. Phoshatidylinositol Turnover

The increased turnover of phosphatidylinositol (PI) in the presence of ACh or CCK was first described by Hokin and Hokin (1955, 1956) in pigeon pancreatic slices. This observation has since been confirmed in the pancreas (Bauduin and Cantraine 1972; Calderon et al 1980) and in other tissues (Michell 1975). Furthermore, the calcium ionophore A23187 does not accelerate degradation of PI under conditions where the ionophore causes changes in other cellular functions (Jones and Michell 1975; Michell 1975). Thus, in tissues in which calcium may mediate the actions of various agonists, the change in PI turnover is thought to preceed calcium mobilization. The PI response seems to correlate with the calcium-mediated secretory process. In the pancreas, cholinergic agents and CCK increase PI turnover while secretin (calcium-independent response) does not affect degradation of PI (Michell 1975).

It has been recognized for some time that phosphatidylinositols, especially diphosphoinositide (DPI) and triphosphoinositide (TPI) have a

high affinity for calcium (Hauser and Dawson 1967) and are therefore

possible calcium storage sites in the membrane. It has been suggested

that activation of muscarinic receptors leads to hydrolysis of PI in

plasma membranes and thus directly or indirectly to calcium release or,

the entry of calcium from the extracellular fluid (Michell 1975).

3. Plasma Membrane Ca^{2+}-ATPases

Calcium is involved in the regulation of a number of cellular

functions including stimulus-secretion coupling (Douglas 1968),

excitation-contraction coupling (Huxley 1973), control of membrane

transport (Porzig 1972) and release of transmitter from motor nerve

terminals (Katz and Miledi 1967). The control of free cytosolic Ca^{2+} is

a necessary requirement for metabolic regulation.

The free calcium concentration in the cell cytoplasm is typically

between 10^{-5} and 10^{-7}M in contrast to the much higher levels of calcium

(approximately 10^{-3}M) in the extracellular spaces. Calcium therefore

penetrates into cells continuously by passive diffusion down its

chemical gradient.

There are various cellular mechanisms that are responsible for

removing calcium from the cytosol: Intracellular calcium can be

sequestered by the mitochondria and endoplasmic reticulum. For the

long-term maintenance of low intracellular free calcium, the

Na^{+}/Ca^{2+} exchange system and the Ca^{2+}-ATPase in the plasma membrane are

the important mechanisms.

It is now apparent that Na^{+}/Ca^{2+} exchange is not an exclusive

feature of excitable cells. It has been demonstrated, not only in nerve

axons and cardiac muscle (DiPolo and Beaugé 1980; Mulins 1979), but also

in nonexcitable cells like liver (Famulski and Carafoli 1982),

intestinal epithelium (Murer and Hildman 1981), kidney basolateral

membranes (Gmaj et al 1979) and exocrine pancreas (Schulz and Heil

1979). The plasma membrane Ca^{2+}-ATPase is also present in excitable

cells such as nerve axons and cardiac muscle (DiPolo and Beaugé 1979)

and cardiac sarcolemma (Caroni and Carafoli 1981). Where the two

systems have been compared directly it appears that they are

complimentary: the exchanger being a low affinity, high-capacity

transporting system, and the Ca^{2+}-pumping ATPase, a higher affinity but

low capacity system (Penniston 1982).

A Ca^{2+}-ATPase associated with Ca^{2+}-transport was first demonstrated

in erythrocyte membranes (Schatzmann 1966; Schatzmann and Vincenzi

1969). Since then a high affinity Ca^{2+}-ATPase has been described in a

wide variety of cells: These include, granulocytes (Schneider et al

1979), axons (DiPolo and Beaugé 1979), macrophages (Lew and Stossel

1980), liver (Lotersztajn et al 1981), corpus luteum (Verma and

Penniston 1981), Lymphocytes (Lichtman et al 1981), heart (Coroni and

Carafoli 1981), brain synaptosomes (Gill et al 1981), adipocytes

(McDonald et al 1982), islet cells (Kotagal et al 1982), thyroid (Kasai

and Field 1982) and bone cells (Shen et al 1983). The human erythrocyte

Ca^{2+}-ATPase is by far the most studied and has been regarded as the

prototype by which other such ATPases are judged. Its properties will

therefore be described in detail here. Several lines of evidence

suggest that the $(Ca^{2+}+Mg^{2+})$-ATPase originally described by Dunham and

Glynn (1961) is the enzymatic expression of the Ca^{2+}-pump, extruding Ca^{2+} from the erythrocyte cytoplasm (Schatzmann and Vincenzi 1969). Recently, this has been unequivocally confirmed by purification of the enzyme and demonstration of its Ca^{2+}-transport properties in reconstituted liposomes (Carafoli et al 1982).

The mechanism by which energy from the hydrolysis of ATP is utlized to transport Ca^{2+} against an electrochemical gradient is not clearly understood. In recent years considerable progress has been made in the elucidation of the partial reactions of the Ca^{2+}-ATPase in erythrocyte membranes. The reaction proceeds through a phosphorylated-protein intermediate [EP], a labile acyl-phosphate which is sensitive to hydroxylamine and basic conditions. Formation of this intermediate is enhanced by Ca^{2+} (Knauf et al 1974; Katz and Blostein 1975; Rega and Garrahan 1975) in a manner similar to the concentration dependence of the Ca^{2+} stimulation of the ATPase activity (Katz and Blostein 1975; Rega and Garrahan 1975; Szász et al 1978). The phosphorylated intermediate is a 150,000 dalton protein (Knauf et al 1974; Katz and Blostein 1975; Wolf et al 1977) which is chemically distinct from the $(Na^{+}+K^{+})$-ATPase phosphoenzyme of molecular weight around 100,000 daltons (Knauf et al 1974; Katz and Blostein 1975). Whereas the substrate for the Ca^{2+} pump was initially proposed to be free ATP (Rega and Garrahan 1975; Schatzmann 1977; Richards et al 1978). Graf and Penniston (1981) proposed that at low ATP concentrations, CaATP was the substrate. Substrate binding results in the formation of an initial phosphoenzyme, E_1P. The K_d for the formation of E_1P (1-6 μM) is similar to the K_d

for ATP hydrolysis (2-5 μM) (Richards et al 1978). Phosphorylation in

the presence of Ca^{2+} occurs in the absence of added Mg^{2+} (Rega and

Garrahan 1975). However, Ca^{2+} dependent formation of the phosphoenzyme

is accelerated by Mg^{2+} (Schatzmann and Bürgin 1978; Rega and Garrahan

1978; Enyedi et al 1980). The major effect of Mg^{2+} appears to be the

conversion of E_1P to a second state, E_2P (Rega and Garrahan 1975;

Garrahan and Rega 1978). The E_2P intermediate is more reactive than E_1P

and can undergo rapid hydrolysis to release Pi (Rega and Garrahan 1978),

consistent with the higher turnover of Ca^{2+}ATPase in the presence of

Mg^{2+} (Katz and Blostein 1975; Garrahan Rega 1978). The final step in

the sequence is the conversion of E_2 to E_1, a step which is believed to

be regulated by calmodulin (Muallem and Karlish 1980). The actual

transport of Ca^{2+} across the plasma membrane is thought to be through

conformational changes in the translocating protein (Schatzmann and

Bürgin 1978). Figure 1 describes the full reaction sequence.
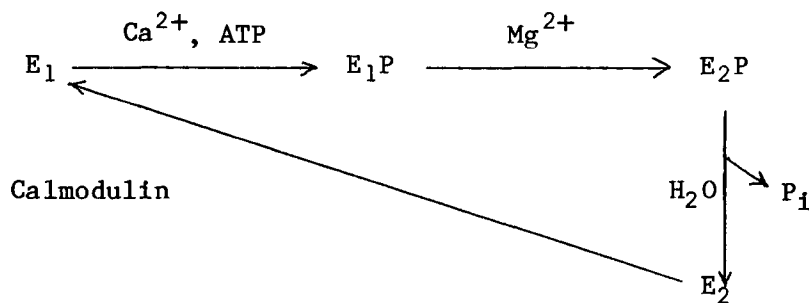
Figure 1. Reaction sequence of $(Ca^{2+} + Mg^{2+})$-ATPase proposed by
Muallem and Karlish (1980, 1981)

Although there is general agreement on the role of Ca^{2+}, Mg^{2+} and

ATP in the regulation of the erythrocyte Ca^{2+}-ATPase, considerable

discrepancy exists in the literature as to the kinetic properties of the enzyme. Analysis of the Ca^{2+} activation of the Ca^{2+}-ATPase revealed both a high and low affinity activity (Horton et al 1970; Schatzmann and Rossi 1971; Scharff 1972). The Ca^{2+} dissociation constant (K_d) for these activities was estimated to be 4 μM and 100 μM, respectively (Schatzmann and Rossi 1971; Scharff and Foder 1977). Scharff (1972) and Schatzmann (1973) however concluded that the low affinity activity was an artifact due to the conversion of the high Ca^{2+} affinity form by the use of chelating agents such as EGTA and EDTA in the membrane preparation. This conclusion is at variance with the results of Horton et al. (1970), who used membranes prepared in the absence of chelating agents and demonstrated the presence of high and low affinity Ca^{2+}-ATPase activities. The variability in the apparent affinities of the enzyme may depend on whether calmodulin is associated or dissociated from the Ca^{2+} pump due to the presence or absence of Ca^{2+} in the hemolysing buffer (Katz et al 1979). Also it has been observed that the presence of Ca^{2+} chelating substances such as EGTA and EDTA in the assay medium will affect the kinetics of the Ca^{2+}-ATPase (Sarkadi et al 1979; Al-Jobore and Roufogalis 1981a).

Attempts to determine the stoichiometry of the erythrocyte Ca^{2+} pump have yielded conflicting results. Schatzmann and Vincenzi (1969) and Schatzmann (1973) suggested a calcium to Pi ratio of one. Quist and Roufogalis (1975) and Sarkadi et al (1977) showed that maximum inhibition of Ca^{2+} transport by lanthanum was associated with only 50% inhibition of ATPase activity. They concluded that La^{3+}-insensitive

ATPase activity was not associated with Ca^{2+} transport and should be subtracted from the total ATPase activity yielding a stoichiometric estimate of 2 Ca^{2+} ions pumped per Pi released. Larsen et al (1978a) using an ion-selective electrode method for rapid, and continuous assessment of Ca^{2+} efflux from resealed red cell "ghosts" prepared from outdated blood did not find a significant La^{3+}-insensitive Ca^{2+}-ATPase activity and hence reported a stoichiometry of 1:1.Recently, reports from various laboratories clearly point to the fact that the stoichiometry of the pump can vary depending on the concentration of effectors used (Sarkadi 1980; Larsen et al 1981; Akyempon and Roufogalis 1981), which may explain the differences previously encountered in various laboratories.

Very little information exists in the literature concerning the properties of other plasma membrane Ca^{2+}-ATPases. In contrast to the majority of high affinity ATPases that require Mg^{2+}, the ATPases of the plasma membranes from rat corpus luteum (Verma and Penniston 1981), rat liver (Lotersztajn et al 1981) and pancreatic islets (Kotagal et al 1982) do not seem to have a similar requirement. By the use of the metal ion chelator trans-cyclohexane -1,2-diamine-N,N,N',N'-tetraacetic acid (CDTA) it was possible to show an apparent requirement for added Mg^{2+} in these systems, suggesting that endogenous Mg^{2+} was difficult to remove from these membranes but was still required for the activity of the enzyme.

In numerous other systems such as the heart (Caroni and Carafoli 1981), synaptosomes (Gill et al 1981), squid nerve (DiPolo and Beaugé

1979), adipocytes (McDonald et al 1982) and macrophages (Lew and Stossel 1980) the presence of a high affinity Ca^{2+}-ATPase and associated Ca^{2+}-transport have been observed. In the liver such a correlation has not yet been demonstrated (Lotersztajn et al 1981).

4. Regulation of Plasma Membrane Ca^{2+}-ATPases

A. Role of Calmodulin

Bond and Clough (1973) first reported that a non-hemoglobin protein present in the hemolysates of human erythrocytes increased the Ca^{2+}-ATPase activity of isolated erythrocyte membranes. Following purification by a number of laboratories (Luthra et al 1977; Jarrett and Penniston 1977, 1978) this factor was shown to be indistinguishable from the purified brain phosphodiesterase or adenylate cyclase activator protein (Gopinath and Vincenzi 1977; Jarrett and Penniston 1977). Calmodulin, as it was termed by Cheung et al (1978) also stimulated Ca^{2+} uptake into inside-out red cell vesicles (Hinds et al 1978; Larsen and Vincenzi 1979; MacIntyre and Green 1978).

Calmodulin has the unique property of regulating a variety of cellular functions. It is involved in synaptic transmission (DeLorenzo et al 1979), calcium induced insulin release and intestinal secretion (Ilundain and Naftalin 1979). Other cellular processes regulated by calmodulin are calcium transport into sarcoplasmic reticulum (Katz and Remtulla 1978) and synaptosomes (Sobue et al 1979), cell multiplication (Whitfield et al 1979) and fertilization of oocytes by spermatozoa

(Jones et al 1978). Calmodulin exerts its effects by stimulating a
number of enzymes including calcium transport ATPases, phosphodieste-
rase, adenylate cyclase, myosin light chain kinase, phosphorylase B
kinase and several other protein kinases (Scharff 1981).

Calmodulin belongs to a group of homologous Ca^{2+}-binding proteins
such as troponin C, parvalbumin, myosin light chains, vitamin D
inducible protein and calsequestrin. Calmodulin is unique among this
group of proteins in that it is ubiquitously distributed in most
eucaryotic cells and shows lack of tissue or species specificity
(Stevens et al 1976). In mammalian tissues, it is highest in brain and
testis (Watterson et al 1976; Dedman et al 1977). The richest source
reported to date is the electroplax of the electric eel where calmodulin
constitutes 2% of the total protein (Childers and Siegel 1975). In all
tissues, calmodulin is distributed between the soluble and particulate
fractions (Smoake et al 1974). Calmodulin binding to particulate
fractions requires Ca^{2+} and appears to occur at specific sites
(Vandermeers et al 1978). Binding is saturable, reversible and
temperature and trypsin-sensitive (Brostrom and Wolff 1981).

Calmodulin is a heat stable acidic globular protein with a molecular
weight of 16,790, as determined from its amino acid sequence (Klee et al
1980). The acidic amino acids, aspartic and glutamic acids constitute
one-third of the total amino acid residues, accounting for its low
isoelectric point (3.5-4.3). It lacks cysteine, hydroxyproline and
tryptophan and has few tyrosine residues. A distinguishing
characteristic is the presence of a trimethyl lysyl residue (Watterson

et al 1980). On the other hand, like the other Ca^{2+} binding protein
such as troponin C (Reid and Hodges 1980) calmodulin exhibits four
internally homologous sequences or domains, each of which contains the
appropriate amino acids forming the helix-loop-helix Ca^{2+} binding
structure (Watterson et al 1980). Based on Kretsinger's hypothesis
(Kretsinger 1976), calmodulin binds four Ca^{2+} ions and the four Ca^{2+}
binding sites correspond to the four domains. It has been shown that of
the four Ca^{2+} binding sites, three sites exhibit high affinity for
calcium (K_d = 0.2 μM) but low affinity for Mg^{2+} (k_d = 140 μM). The
fourth site has similar affinities for both Ca^{2+} and Mg^{2+} (Wolff et al
1977). A variety of data supports the conclusion that Mg^{2+} and other
divalent cations compete for the Ca^{2+} binding sites on calmodulin.
Direct binding studies indicated that 4 moles of Mg^{2+} or Mn bind per
mole of calmodulin and are displaced by Ca^{2+} (Wolff et al 1977). Also,
Mg^{2+} increases the apparent K_d for Ca^{2+} of various
calmodulin-dependent enzymes (Katz et al 1979; Brostrom et al 1977).

Calmodulin regulation of enzyme activity has generally been found to
require Ca^{2+} although Ca^{2+}-independent calmodulin effects have been
reported in systems such as Bordetella pertusis adenylate cyclase
(Greenlee et al 1982; Kilhoffer et al 1983), pig lung cyclic GMP
dependent protein kinase (Yamaki and Hidaka 1980) and histone
dephosphorylation by liver phosphoprotein phosphatases (Khandelwal et al
1980). The mechanism of activation in these system is not fully
understood.

Regulation of Ca^{2+}-dependent enzyme (E) by calmodulin (C) is

believed to proceed by 2 sequential, fully reversible mass action

equations (Brostrom and Wolff 1981).

$$nCa^{2+} + C \rightleftharpoons (Ca^{2+})n\ C \tag{1}$$

$$x(Ca)nC + E_{inactive} \rightleftharpoons [(Ca^{2+})n\ C]x\ E_{active} \tag{2}$$
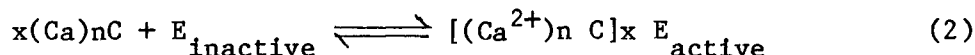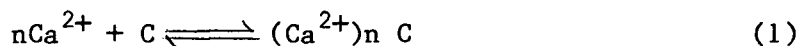
Where n equals the number of binding sites which must be occupied by

Ca^{2+} to convert calmodulin to an activating form for the enzyme and x

equals the unknown number of $(Ca^{2+})n$ calmodulin complexes. In this

model, Ca^{2+} first interacts with calmodulin to form a complex capable of

activating the enzyme (Eqn 1). This complex then associates with the

inactivated form of the enzyme forming an activated ternary complex (Eqn

2). Saturation of calmodulin with Ca^{2+} results in a conformational

change involving an increase in the α-helical content from 28% to 42%

(Brostrom and Wolff 1981). Tanaka and Hidaka (1980) have shown that the

conformational change produced in calmodulin by binding of Ca^{2+} to the

high affinity sites exposes hydrophobic groups which in turn appear to

be involved in activating calmodulin-dependent enzymes. Thus, both the

acidic nature of calmodulin and its Ca^{2+}-inducible hydrophobic

properties seem to be important in regulating calmodulin dependent

enzymes. In the red blood cell, calmodulin stimulates the decomposition

of the EP complex of the Ca^{2+}-ATPase, in a manner similar to Mg^{2+}

(Jeffery et al 1981).

Even though calmodulin is known to regulate a majority of plasma

membrane Ca^{2+}-ATPases a clear demonstration of this effect is lacking in

the Ca^{2+}-ATPases of rat corpus luteum (Verma and Penniston 1981) and rat liver (Lotersztajn et al 1981). These systems did not respond to added calmodulin even after washing of the membranes with EGTA (Verma and Penniston 1981) or purification of the enzyme on DEAE-cellulose (Lotersztajn et al 1981). The possibility remains though that endogenous calmodulin is bound such that it could not be removed by these treatments. However the existence of other modes of regulation cannot be ruled out. Indeed Lotersztajn et al (1981) have reported that rat liver plasma membrane Ca^{2+}-ATPase is activated by an endogenous protein activator which is distinct from calmodulin in a number of properties. In contrast, the heart sarcolemmal Ca^{2+}-ATPase is regulated by both calmodulin and a cyclic AMP-dependent phosphorylation process (Caroni et al 1982).

B. Role of Acidic Phospholipids, Long-chain Fatty Acids and Controlled Proteolysis

It was first shown by Ronner et al (1977) and later by Al-Jobore and Roufogalis (1981b) that erythrocyte Ca^{2+}-ATPase dissolved from the membrane by detergents could be activated by a variety of acidic phospholipids and by polyunsaturated fatty acids. Niggli et al (1979) observed that erythrocyte Ca^{2+}-ATPase purified in the presence of phosphatidylserine exhibited high specific activity but could not be stimulated by calmodulin. In contrast, Gietzen et al (1980a) isolated the purified enzyme in the presence of phosphatidycholine and reported an eight-to nine-fold stimulation of the purified enzyme by calmodulin.

It soon became apparent that acidic phospholipids and unsaturated fatty acids could mimic the effect of calmodulin. Extensive studies done on the purified erythrocyte Ca^{2+}-ATPase (Niggli et al 1981) indicated that it is stimulated by a variety of acidic phospholipids (phosphatidylserine, cardiolipin, phosphatidylinositol and phosphatidic acid). Unsaturated fatty acids (oleic and linoleic acid) had a similar effect. On the other hand, neutral phospholipids (phosphatidylcholine, sphingomyelin and phosphatidylethanolamine) had no effect on the Ca^{2+}-ATPase activity (Niggli et al 1981). It has now been found that diphosphoinositide (DPI) and triphosphoinositide (TPI) are also powerful activators of the reconstituted Ca^{2+}-ATPase (Carafoli et al 1982); the significance of the finding lies in the fact that they activate at a much lower concentration than acidic phospholipids and fatty acids, and could function as a physiological regulator of the enzyme.

Taverna and Hanahan (1980) and Sarkadi et al (1980) observed that limited proteolysis of erythrocyte membranes was another method of activation of the Ca^{2+}-ATPase. Maximum proteolytic activation resulted in the removal from the ATPase of a fragment of M_r about 30,000 (Sarkadi et al 1980). Since the treatment removed the sensitivity to calmodulin, Sarkadi et al (1980) suggested that the 30,000 M_r fragment is the calmodulin receptor of the enzyme molecule. On the contrary, studies with purified Ca^{2+}-ATPase indicated that calmodulin binding capacity was lost upon removal of a 10,000 M_r polypeptide by tryptic digestion (Carafoli et al 1982). The possible explanation is that acidic phospholipids, fatty acids, and the calmodulin-Ca^{2+} complex act

by introducing into the region of the active site of the enzyme a

relatively hydrophobic environment (Carafoli et al 1982). This will

result in a conformational change such that the active site becomes more

accessible. Removing a hypothetical inhibitory polypeptide from the

enzyme molecule by proteolytic treatment would also expose the active

site. Thus in the case of calmodulin, Ca^{2+} would be required to

increase its hydrophobicity (Tanaka and Hidaka 1980) whereas other

treatments would not require Ca^{2+}.

C. Regulation of the Action of Calmodulin and other Activators of Ca^{2+}-ATPase activities

At present, relatively little is known about the factors which

control calmodulin mediated functions. Recent reports that insulin

inhibits Ca^{2+}-ATPase activity in rat adipocytes (McDonald et al 1982)

has generated some interest due to the possibility that insulin may

antagonize calmodulin activation in this system. Evidence for direct

binding of calmodulin to various cellular proteins has also been cited

in the literature. Wang and Desai (1977) isolated and characterized a

calmodulin-binding protein (CaM-BP) from bovine brain: calmodulin

binding to the protein was Ca^{2+}-dependent. Klee et al (1979) suggested

the name calcineurin to refer to the heat labile CaM-BP they isolated

from nerve tissue. Heat stable (Sharma et al 1978) and heat labile (Au

1978) forms of CaM-BPs have been isolated from bovine brain and

erythrocytes, respectively. It was demonstrated that CaM-BP selectively

antagonized calmodulin activation of erythrocyte Ca^{2+}-ATPase and

Ca^{2+}-transport (Au 1978; Larsen et al 1978b). Little is known about the
function or regulation of the levels of CaM-BPs in cells. However, it
is believed that CaM-BPs may represent a means of regulating calmodulin
activity.

The demonstration by Levin and Weiss (1977) that certain
antipsychotic drugs, such as trifluoperazine and related phenothiazines,
blocked calmodulin activation of phosphodiesterase activity resulted in
a major advancement in the investigation of calmodulin-regulated
systems. Subsequently it was shown that phenothiazines also antagonized
calmodulin activation of Ca^{2+}-ATPase and Ca^{2+}-transport in human
erythrocyte membranes (Gietzen et al 1980b; Raess and Vincenzi 1980;
Roufogalis 1981). The binding of trifluoperazine (Levin and Weiss 1977)
and related phenothiazines (Levin and Weiss 1979) to calmodulin is
calcium-dependent. Schatchard plot analysis revealed two types of
trifluoperazine binding sites, of high affinity (K_d = 1-1.5 μM) and
low affinity (K_d = 5 μM). The low affinity sites are
Ca^{2+}-independent, in contrast to the high Ca^{2+} affinity sites (Levin and
Weiss 1979). A similar distribution of sites was found for
chlorpromazine (K_d = 5 μM and 130 μM).

Since the discovery that phenothiazines inhibit calmodulin-dependent
activation of enzyme systems, a variety of compounds have been shown to
antagonize the action of calmodulin. These agents include antipsychotic
agents, antidepressants, local anesthetics, vincristine, rauwolfia
alkaloids, antihypertensives, N-(6-aminohexyl)-5-chloro-1-naphthalene-
sulfonamide (W7), 1-[bis(p-chlorophenyl) methyl]-3-[2,4-dichloro-β-

(2,4-dichlorobenzyloxyl) phenethyl] imidazolinium chloride (R24571 or
calmidazolium), Triton X-100 (Roufogalis 1982) and Compound 48/80
(Gietzen et al 1983) . The most potent compound reported to date is
calmidazolium. The I_{50} for inhibition of calmodulin activation of
phosphodiesterase by this compound is 0.01 μM (Belle 1981). Of the
commonly used drugs, pimozide (l_{50} = 0.7 μM), penfluridol (l_{50} = 2.5
μM), and trifluoperazine (l_{50} = 10 μM) are among the most potent
anticalmodulin drugs (Roufogalis 1982).

Levin and Weiss (1976) noted that the antianxiety agents, medazepam
and chlordiazepoxide, and the antidepressants, amitryptyline,
protriptyline and desipramine, as well as the non-antipsychotic analogs
of phenothiazines, chlorpromazine sulfoxide and trifluoperazine
sulfoxide (Levin and Weiss 1979) are less potent and show less
Ca^{2+}-dependence as inhibitors of calmodulin action. It was therefore
suggested that there is a correlation between calmodulin antagonism and
antipsychotic activity. However, recent findings are not consistent
with this hypothesis: Haloperidol, a member of the butyrophenone class
of clinical antipsychotic agents is 50-fold more potent than
chlorpromazine clinically, yet it has a similar or lower potency as an
antagonist of calmodulin-activated phosphodiesterase (Levin and Weiss
1979; Norman et al 1979) and is ineffective against calmodulin
activation of erythrocyte Ca^{2+}-ATPase (Raess and Vincenzi 1980a).
Another piece of evidence against this hypothesis is the lack of
stereospecificity of calmodulin inhibition in contrast to antisychotic
activity: Roufogalis (1981) demonstrated that all of a series of

chlorpromazine analogs; in which the chlorine substitution was varied from position 1 to 4 on the A ring of the tricyclic nucleus inhibited calmodulin activation of erythrocyte Ca^{2+}-ATPase activity, yet only the compound with chlorine substituted on position 2 showed tranquilizer activity. In view of these discrepancies, the relationship between the antagonism of calmodulin and the clinical efficacy of the antipsychotic agents seems unlikely.

A critical study of the wide variety of anticalmodulin agents by Norman et al (1979) revealed that hydrophobicity is a major factor contributing to their action. Indeed in a diverse series of drugs, these workers found a linear relationship between the logarithm of the potency of antagonism of calmodulin activation of phosphodiesterase and the log octanol: water partition coefficient. The potent calmodulin inhibitors are amphipathic compounds that contain large hydrophobic regions and carry a positive charge at neutral pH. The interactions of these drugs with calmodulin appear to involve two processes: There is the hydrophobic interaction between lipophilic portions of the drug and non-polar regions of calmodulin and an electrostatic interaction occurring between a positively charged group on the drug and a negatively charged acidic residue on calmodulin (Weiss et al 1982).

The specificity of phenothiazines for inhibition of calmodulin-mediated events has been a major concern of many investigators. It is now known that phenothiazines and other anticalmodulin agents also effectively block the activation of phosphodiesterase and erythrocyte Ca^{2+}-ATPase by acidic phospholipids

and unsaturated fatty acids (Gietzen et al 1982). Phenothiazines have been shown to bind to some "calmodulin-like" proteins isolated from Chlamydomonas flagella (Van Eldik et al 1980) and rat heart (MacManus 1981). Phenothiazines also have α-adrenergic and muscarinic receptor blocking activities, as well as specific dopamine and serotonin blocking activities (Roufogalis 1982). Thus despite their usefulness as tools in studying calmodulin activation of various enzyme systems in vitro, the relationship of these effects to their mechanism of action in vivo is uncertain.

5. Objectives of the Present Study

Calcium ions play an important role in the stimulation of enzyme secretion from the exocrine pancreas. It has been proposed that a secretagogue-induced rise in cytosolic free calcium concentration triggers enzyme release from pancreatic acinar cells (Schulz 1980; Williams 1980; O'Doherty and Stark 1982; Dormer 1983). Therefore, the precise regulation of cytosolic free calcium is critical for the physiological function of the acinar cell. In a number of different cell types, a plasma membrane Ca^{2+}-ATPase and associated Ca^{2+}-transport has been shown to be responsible for maintenance of low intracellular free calcium concentrations (Schatzmann and Vincenzi 1969; Caroni and Carafoli 1981; McDonald et al 1982).

A Ca^{2+}-ATPase activity has been described in plasma membrane-enriched fractions prepared from pancreatic tissue homogenates (Milutinovic et al 1977). Various investigators (Forget and Heisler

1976; Lambert and Christophe 1978; Le Bel et al 1980; Martin and Senior 1980) have shown that this enzyme is stimulated by both Ca^{2+} and Mg^{2+} to a similar extent. It was not possible though to determine the exact cellular origin of the plasma membrane fraction in the preparations used since the pancreas is a multicellular organ. Moreover, there is no report on the mode of regulation of this enzyme. Therefore the objectives of the present study are:

1. Determination and characterization of a possible Ca^{2+}-ATPase activity in pancreatic acinar cell plasma membranes.

2. Determination of the mechanism of regulation of this enzyme by investigating the effects of calmodulin, and acidic phospholipids.

3. Characterization of Ca^{2+}-transport processes in vesicles prepared from pancreatic acinar membranes.

4. Determination of the possible relationship of Ca^{2+}-ATPase and Ca^{2+}-transport to amylase secretion by -

   a. investigation of the effect of phenothiazines (anticalmodulin drugs) on amylase release from pancreatic acinar cells and,

   b. identification of the orientation of the catalytic site of the Ca^{2+}-transport process in the intact acinar cell.

MATERIALS AND METHODS

I. MATERIALS

The chemicals and/or proteins were purchased from the following sources:

1. Sigma Chemical Co.

   Collagenase

   Carbamylcholine chloride (Carbachol)

   Pancreozymin

   Secretin

   Soybean trypsin inhibitor Type I-S

   Aprotinin

   Ascorbic acid

   EGTA

   EDTA

   Tris-ATP

   2-Mercaptoethanol

   Cardiolipin

   Phosphatidylserine

   Phosphatidylinositol

   Phosphatidylcholine

   Oleic acid

   Compound 48/80

Tris base

Tris hydrochloride

Triton X-100

Glucose

Lithium dodecyl sulphate

Chlorpromazine

Bovine serum albumin

Sodium azide

Trichloroacetic acid

Hydroxylamine monohydrochloride

Bromphenol blue

2. Amersham

$[\gamma^{32}P]$-ATP (Specific activity = 5-10 x 10^6 DPM/μmole)

$^{45}CaCl_2$(Specific activity = 1.5 x 10^6 CPM/μmole)

3. Calbiochem

Calmodulin

Adenyl 3',5'-adenosine

4. Smith, Kline and French

Trifluoperazine (a gift)

5. Grand Island Biological Co.

Minimum Eagles medium amino acids

Trypan blue

6. BDH Biochemicals

    Sodium dodecylsulphate

    Calcium chloride

7. Bio-Rad

    Acrylamide

    N,N'-methylene-bisacrylamide

    Coomassie Brilliant Blue R-250

    Ammonium persulphate

8. Fisher Scientific Co.

    Sodium chloride

    Sodium phosphate monobasic (NaH_2PO_4)

    Sodium phosphate dibasic (Na_2HPO_4)

    Lanthanum chloride

    Hydrochloric acid (HCl)

    Glacial acetic acid (CH_3COOH)

9. MCB Manufacturing

    Asolectin

10. Boehringer-Mannheim

    Hyaluronidase

II. METHODS

1. Preparation of Dispersed Rat Pancreatic Acinar Cells

The cells were isolated by the method of Kondo and Schultz (1976a) as modified by Chauvelot et al (1979). Pancreas was obtained from rats, killed by a blow to the neck. All the steps in the isolation of the cells were done using a Krebs-Ringer-bicarbonate (KRB) solution equilibrated with 95% O_2 and 5% CO_2 containing 15 mM glucose and 0.36 mg soybean trypsin inhibitor per ml.

(a) First digestion: the pancreas was trimmed free of fat and mesentery and 10 ml of the enzyme mixture (0.9 mg of crude collagenase/ml and 1.5 mg hyaluronidase/ml in KRB solution containing 0.1 mM Ca^{2+} and 1.2 mM Mg^{2+}) was inoculated into the interstitium of the tissue by means of a 1 ml syringe with a 25 gauge 5/8 needle. The distended gland and excess enzyme solution was transferred to a siliconized 100 ml Erlenmeyer flask, equilibrated with 95% O_2 and 5% CO_2 and incubated at 37°C for 15 min. with agitation at 108 oscillations/min.

(b) Removal and replacement of divalent cations: the supernatant was decanted and the tissue then incubated twice for 5 min. at 37°C in 20 ml of KRB solution without Ca^{2+} and Mg^{2+} and containing 2 mM EDTA. Divalent cations were subsequently added back by briefly washing the tissue twice with 20 ml of KRB solution containing 0.1 mM Ca^{2+} and 1.2 mM Mg^{2+}.

(c) Second digestion: fresh collagenase (10 ml with 0.1 mM Ca^{2+}

and 1.2 mM Mg^{2+}) at concentration of 1.5 mg/ml was added and the cells

incubated for 25 - 30 min. at 37°C.

(d) Final dissociation and harvesting of the cells: The cells were

dissociated by sequential passage (two times) through Eppendorf 1 ml

pipettes with tips cut to diameters 3 mm and 1.5 mm respectively. The

isolated cells were filtered two times through nylon filter (149 μm,

Cole-Parmer) and layered in centrifuge tubes over 10 ml columns of KRB

solution with 0.1 mM Ca^{2+} and 1.2 mM Mg^{2+} containing 4% bovine serum

albumin. The tubes were centrifuged at 50 x g for 6 min. The cells

were resuspended in 15 ml of the same solution and counted using a

hematocytometer. The cells were again centrifuged and the pellet was

finally suspended in KRB solution with 0.1 mM Ca^{2+}, 1.2 mM Mg^{2+}, 10 mM

glucose, 10 mg/ml of bovine serum albumin, 0.36 mg/ml trypsin inhibitor

and kept at room temperature.

2. Determination of the Insulin Content of Enzymatically Dispersed Pancreatic Cells

Enzymatically dispersed pancreatic cells and the pellet obtained

from the final centrifugation step described above were washed twice in

a 20 mM Tris-HCl buffer (pH 7.5) containing 154 mM NaCl and then

homogenized in the same buffer containing 0.1% Triton X-100 and 5 M

urea. The cellular homogenate was centrifuged at 3000 x g for 30 min

and the supernatant assayed for insulin using an insulin

[^{125}I]radioimmunoassay kit (Becton Dickinson).

3. Cytochemical Staining of Pancreatic Acinar Cells

The pellet from the 4% BSA centrifugation step was embedded into a piece of muscle tissue. The tissue was mounted on a cork disc by means of Tagacant gum and quickly frozen in isopentane (2-methylbutane) sitting in a flask of liquid nitrogen. The frozen tissue was then kept at -30°C for 3 hours following which it was sectioned in a cryostat (Demon/IEC Division) and mounted on glass slides. Sections were stained in Cox-Lillie-Meyer haematoxylin for 3-6 min, washed three times in tapwater and then stained for another minute with 1% eosin. This was followed by dehydration through alcohols, cleared in xylene and then mounted.

4. Measurement of Amylase Release from Acinar Cells

Cells were resuspended in KRB solution containing 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 10 mM glucose, 10 mg/ml bovine serum albumin and 0.12 mg/ml soybean trypsin inhibitor at a concentration of 0.5×10^6 cells/ml in a final volume of 2.5 mls. The cells were incubated in a 10 ml siliconized Erlenmeyer flask at 37°C in the presence or absence of secretagogues and other drugs. At different periods of incubation, 100 µl aliquots were taken into microcentrifuge tubes and centrifuged (Eppendorf Centrifuge Model 3200) at 10,000 rpm for 45 sec. Aliquots of 50 µl were transferred to 950 µl of hypotonic medium consisting of 1 mg/ml bovine serum albumin and 1 mg/ml sodium dodecyl sulphate (SDS) in 10 mM phosphate buffer pH 7.4. Then a 50 µl aliquot was assayed for amylase using the Phadebas (Pharmacia) Kit. To determine total amylase,

50 μl of the cell suspension was added to 9.95 ml of hypotonic medium and vortexed vigorously. A 50 μl aliquot was then assayed for amylase. Amylase released was expressed as a percentage of total amylase:

$$\% \text{ Release} = \frac{\text{Units amylase } t_n - \text{Units amylase } t_o}{\text{Units amylase in cell lysate}} \times \text{Dilution Factor} \times 100$$

where t_n = time at end of incubation

t_o = zero time sample

5. Preparation of Pancreatic Acinar Plasma Membranes

Plasma membranes were prepared from acinar cells according to the method of Svoboda et al (1976) with slight modifications. The cells were homogenized in a glass-teflon homogenizer at 900 rev/min for 1 min in a cold buffer (2-4°C) consisting of 10 mM Tris-HCl (pH 7.4), 0.3 M sucrose, 5 mM 2-mercaptoethanol, Aprotinin (500 kallikrein inactivator units/ml), 1 mM EDTA and 2 mM $MgCl_2$. The homogenate was further diluted with 120 ml of buffer, filtered through two layers of cheesecloth and centrifuged at 180 x g for 10 min. The supernatant was centrifuged again at 15000 x g for 15 min and the resulting pellet was resuspended in 15 ml of buffer containing 2 mM EDTA. Then 6 ml of this suspension was layered on top of a discontinuous sucrose density gradient of 27%, 35% and 38% (w/w) sucrose and centrifuged at 25,000 rpm for 3 h in a Beckman Ultracentrifuge (Model L5-50) with SW 27 rotor. Various fractions were aspirated, diluted with sucrose-free buffer and

centrifuged at 70,000 x g for 35 min. The final pellet was resuspended in sucrose-free buffer and used immediately or stored frozen at -80°C.

6. Treatment of Membranes with EDTA

The membrane preparation (1-2 mg/ml) was diluted with an equal volume of buffer containing 1 mM Tris EDTA (pH 8.0) and incubated for 30 min at 37°C. After cooling on ice, the suspension was centrifuged at 20,000 x g for 20 min. The pellet was washed twice and resuspended in the original volume of buffer.

7. Measurement of Divalent Cations in Membrane Preparations

Calcium and magnesium were extracted from membrane preparations by the method of Sparrow and Johnstone (1964). This involved treating the membrane preparations with a solution of 0.6 M trichloroacetic acid (TCA): glacial acetic acid mixture, 1 mM EDTA and 0.5 mM $LaCl_3$. The suspension was centrifuged at 20,000 x g for 35 min and the supernatant was collected for atomic absorption spectrometry in a Techtron AA5 spectrophotometer at 422.8 nm, using nitrous oxide-acetylene for calcium or at 285.2 nm, using acetylene for magnesium. The standards contained identical concentrations of TCA: glacial acetic acid mixture, $LaCl_3$ and EDTA; the standard curve was linear from 0-20 μM.

8. Determination of Calmodulin Content of Plasma Membrane Preparations

Plasma membrane preparations (2 mg/ml) were boiled for 5 min in the presence of 0.2 mM EGTA. After cooling on ice, calcium was added to

chelate the EGTA and the solution was centrifuged at 70,000 x g for 30 min. The supernatant was then assayed for calmodulin using a calmodulin [^{125}I]radioimmunoassay kit (New England Nuclear).

9. Enzyme Assays

a. Measurement of Ca^{2+}-ATPase Activity: Ca^{2+}-ATPase activity was determined in a 0.25 ml medium containing 48 mM Tris-HCl pH 7.4, 0.06 mM EGTA [Ethyleneglycolbis (β-aminoethylether)-N,N'-tetraacetic acid], 0.1 mM ouabain, 2 mM NaN$_3$, 0.07 ml of membrane preparation (0.7 - 1.0 mg/ml) and the desired free Ca^{2+} and/or Mg^{2+} concentration. Calmodulin and/or phenothiazines were added to this medium when required. After preincubation for 15 min at 37°C, the assay was started by the addition of 25 μl of 5 mM ATP, and the reaction was terminated after 15 min by taking 210 μl aliquots into 0.5 ml of 3% (w/v) sodium dodecylsulfate (SDS). ATPase activity was measured by determining Pi released by an automated Fiske and Subbarow (1925) procedure described by Raess and Vincenzi (1980b). Under the assay conditions, the enzyme activity was linear for at least 30 min.

b. Measurement of (Na$^+$ + K$^+$)-ATPase Activity: (Na$^+$ + K$^+$)-ATPase was assayed in a solution containing 75 mM Tris-HCl buffer pH 7.4, 100 mM NaCl, 20 mM KCl, 3 mM MgCl$_2$, 3 mM ATP, 0.1 mM EGTA and in the presence or absence of 1 mM ouabain. Inorganic phosphate released after 20 min incubation was determined as for Ca^{2+}-ATPase. (Na$^+$ + K$^+$)-ATPase was defined as that activity which was inhibited by ouabain.

c. Measurement of 5'-Nucleotidase Activity: 5'-Nucleotidase activity was determined in a solution containing 100 mM Tris-HCl (pH

8.5), 10 mM MgCl$_2$, 10 mM 5'-AMP and 200 mM KCl. 5'-Nucleotidase

activity in the presence of 3'-AMP was subtracted from the total

5'-Nucleotidase in the presence of 5'-AMP to account for nonspecific

phosphatase activity.

    d. Measurement of Cytochrome c Oxidase Activity: Cytochrome c

oxidase activity was measured spectrophotometrically using the method of

Wharton and Tzagaloff (1967). To each of 2 cuvettes, 100 μl of 10 μM

potassium phosphate buffer, pH 7.0, 70 μl 1% ferrocytochrome c, and 0.83

ml of water was added. The ferrocytochrome c was reduced with ascorbic

acid and dialyzed overnight prior to its addition to the reaction

medium. Ten μl of 0.1M potassium ferrocyanate was added to the "blank"

cuvette in order to oxidize the ferrocytochrome present. Both cuvettes

were incubated for 10 minutes at 37°C at which time the reaction was

initiated by the addition of 10 μl of the membrane preparation

(approximately 5 μg protein) to the "reaction" cuvette. The decrease in

absorbance at 550 nm was used as an index of the rate of oxidation of

ferrocytochrome c and was monitored for 3 minutes. Cytochrome c Oxidase

activity of the membrane preparation was determined using the following

first order rate equations:

$$k = \ln \frac{(\text{absorbance at time 0}) \text{ min}^{-1}}{(\text{absorbance at 1 minute})}$$

$$\text{Specific Activity} = k \frac{(\text{concentration of cytochrome c})}{(\text{concentration of protein})}$$

The specific activity was expressed as nmole of cytochrome c oxidized

per mg protein per minute.

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e. Measurement of Lactate Dehydrogenase Activity: Acinar cell
suspensions (10^6 cells/ml) or cells that had been disrupted by freezing
and thawing were centrifuged (Eppendorf Centrifuge Model 3200) at 10,000
rpm for 45 sec and the supernatant assayed for lactate dehydrogenase
activity.

Lactate dehydrogenase activity was measured spectrophotometrically
using the method of Kornberg (1955): to a 3-ml capacity cuvette was
added 0.1 ml of 10 mM sodium pyruvate, 0.1 ml of 2 μM NADH, 1.0 ml of
100 mM phosphate buffer (pH 7.4) and 1.75 ml water. The 'blank' cuvette
was similarly treated except that 0.1 ml buffer was added instead of
NADH. Both cuvettes were incubated for 10 min at 37°C and the reaction
was started by the addition of 50 μl of cell extract. The decrease in
absorbance at 340 nm was used as an index of the rate of oxidation of
NADH and was monitored for 3 min. One unit of enzyme is defined as that
amount which causes an initial rate of oxidation of 1 μmole of NADH per
minute using an extinction coefficient of 6.22. Specific activity was
expressed as μmoles per milligram of protein.

10. Preparation of Phospholipid Dispersions

Aliquots of chloroform solutions of the lipids were evaporated to
dryness under a stream of nitrogen and then reconstituted in 48 mM
Tris-HCl (pH 7.4) by sonication for 3 min in a Braunsonic 1510
ultrasonicator at maximum power. The translucent solution obtained was
centrifuged at 3000 x g for 30 min to remove small metal particles and
large aggregates of phospholipids.

11. Measurement of Ca^{2+}-Transport Activities

a. Measurement of Calcium Efflux from Plasma Membrane Vesicle

Preparations: Vesicles were prepared from membrane preparations by the method of Schulz and Heil (1979) except that 0.3 M sucrose was used instead of mannitol since it was observed that mannitol interfered with the colorimetric determination of inorganic phosphate. For preloading, plasma membranes were homogenized 5 times with a hand homogenizer and vesiculated in a medium containing 0.3 M sucrose, 20 mM Tri-HCl buffer (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 2 mM NaN_3, 5 mM Tris ATP, the desired free Ca^{2+} concentration, $^{45}CaCl_2$ (10 μCi/mmol) and kept at 4°C for 18 hours. To determine Ca^{2+} efflux; 50 μl of the preloaded vesicles (2-3 mg/ml) were transferred to 1.45 ml of a non-radioactive sucrose buffer and incubated at 37°C. At various timed intervals, aliquots of 50 μl were filtered through presoaked 0.45 μm membrane filters (Millipore Co.) under vacuum, and the filters washed with two 5 ml portions of sucrose buffer containing 1.2 mM $LaCl_3$. The dried filters were then transferred to vials and the radioactivity present determined by liquid scintillation spectrophotometry in Aquasol (New England Nuclear). Blanks in the absence of pancreatic membranes were determined in a similar manner and any activity obtained subtracted from the experimental values.

b. Measurement of Ca^{2+}-Uptake by Plasma Membrane Vesicle

Preparations: Vesicles were prepared in 0.3 M sucrose as described above. The sealed vesicles were then suspended in a sucrose buffer containing 20 mM Tris HCl-buffer (pH 7.4), 100 mM KCl, 0.1 mM $MgCl_2$, 2

mM NaN_3, the desired free Ca^{2+} concentration, $^{45}CaCl_2$ (10 Ci/mmole) and

5 mM Tris ATP in a total volume of 1.0 ml. Because of the high

non-specific binding of calcium to the membrane vesicles, the reaction

at 37°C was started by the addition of $^{45}CaCl_2$. At timed intervals, 50

μl aliquots were transferred to 1 ml ice-cold sucrose buffer containing

2 mM EGTA before filtration. Subsequent steps are similar to those

described above. It was observed that the EGTA treatment which removes

bound Ca^{2+} resulted in higher Ca^{2+} uptake rates.

12. Calmodulin Gel Overlay Technique

The calmodulin gel overlay technique was performed using a

modification of the procedure used by Carlin et al (1981): Following

SDS gel electrophoresis (200 μg protein/lane, 5-20% gradient) of

pancreatic plasma membrane fractions, the gels were washed in a solution

of 25% (v/v) isopropanol and 10% (v/v) acetic acid to remove SDS. The

solution was changed four times in a 2 hour period. Following two, 5

min incubations in distilled water, the gels were soaked for 30 min in a

denaturing buffer (0.15 M NaCl, 20% (v/v) glycerol, 1 mM magnesium

acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine serum

albumin, 50 mM Tris-HCl, pH 7.5) supplemented with 6 M guanidine-HCl.

The gels were then continuously washed for at least 24 hours in the

above buffer, without guanidine-HCl, prior to equilibration for 12 hours

in buffer A (0.15 M NaCl, 1 mM magnesium acetate, 1 mM $CaCl_2$, 1 mM

dithiothreitol, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 50 mM

Tris-HCl, pH 7.5). The gels were then soaked in buffer A containing

^{125}I-labeled calmodulin (240 nM, $10^4 - 10^5$ cpm/ pmol, 50 ml/slab) for 24 hours. Non-specifically bound calmodulin was removed by continuous washing for 24 hours in buffer A. Control experiments were performed in which 5 mM EGTA was included in the final wash solutions. The gels were dried and then subjected to autoradiography. To determine molecular weights, the lanes, containing appropriate standards, were cut from the rest of the gel immediately following electrophoresis and stained by Coomassie blue. R_F values were used to assign molecular weights to the calmodulin-labeled bands.

13. Measurement of Acyl-Phosphoprotein Formation

Membrane-ATPase phosphoprotein was measured by the method of Katz and Blostein (1975). Pancreatic acinar plasma membranes (130 μg/100 μl final incubation volume) were incubated for 15s at 10°C in a medium containing 48 mM Tris-HCl (pH 7.4), 0.1 mM ouabain, 2 μM [γ-^{32}P] ATP (1000-3000 cpm/pmol), 60 μM EGTA in the presence and absence of 0.4 mM magnesium acetate. When Ca^{2+} was added it was present in a final concentration of 80 μM free. The reaction was started by the addition of membranes to the reaction medium. After 15s the reaction was terminated by the addition of 45 μl of 'stop' solution consisting of 50 mM Tris phosphate buffer (pH 6.5), 5% lithium dodecyl sulphate (LiDS), 30% urea, 0.5 mM dithiothreitol and 0.002% bromphenol blue as the tracking dye. To determine the stability of the phosphoprotein formed, some samples were treated with 0.6 M hydroxylamine in 0.8 M sodium acetate (pH 5.2) for 10 min at 22°C before the addition of the

'stop' solution. The samples (60 µl per lane) were then analyzed by acid-polyacrylamide gel electrohoresis.

14. Phosphorylation of Endogeneous Acinar Membrane Proteins

Phosphorylation of endogenous substrate proteins was carried out at 30°C in a 100 µl reaction mixture containing 130 µg membrane proteins, 0.1 mM dithiothreitol, 10 mM magnesium acetate, 0.5 mM [γ^{32}P] ATP (1000-3000 cpm/pmol), 20 mM Tris-HCl buffer (pH 7.0) in the presence or absence of 90 µM Ca^{2+}, 2.2 mM EGTA or 100 nM calmodulin. After 5 min of incubation, the reaction was terminated by the addition of 45 µl of 'stop' solution consisting of 50 mM Tris phosphate buffer (pH 6.8), 5% SDS, 30% urea, 0.5 mM dithiothreitol and 0.002% bromphenol blue as the tracking dye. The samples (60 µl/lane) were then analyzed by SDS polyacrylamide gradient gel electrophoresis.

15. Polyacrylamide Gel Electrophoresis and Autoradiography of Phosphorylated Plasma Membrane Preparations

a. **Gradient Gel Electrophoresis:** Polyacrylamide gradient (5-20%) gels of 1.5 mm thickness were cast according to the method of Laemmli and Favre (1973). The 5% 'resolving' gel consisted of 3.33 ml of acrylamide-methylene-bisacrylamide (30%: 0.8%) mixture, 375 mM Tris-HCl (pH 8.8), 1.35% glycerol, 0.1% SDS, 0.025% tetraethylmethylenediamine (TEMED) and 0.4 ml ammonium persulphate (15 mg/ml) in a total of 20 ml. The 20% 'resolving' gel contained 13.33 ml of acrylamide-methylene-bisacrylamide (30%: 0.8%) mixture, 375 mM Tris-HCl buffer (pH 8.8),

5.75% glycerol, 0.1% SDS, 0.05% TEMED and 0.3 ml ammonium persulphate (15 mg/ml) in a total of 20 ml. The 5% and the 20% 'resolving' gel solutions were immediately added to the gel chamber with the aid of a gradient former. About 2 ml of buffer was carefully layered on top of the gel and the gel was allowed to polymerize for 12 hours at room temperature. The buffer was decanted prior to the addition of the 'stacking' gel.

The 'stacking' gel (5%) consisted of 1.25 ml of acrylamide-methylene-bisacrylamide (30%: 0.8%) mixture, 315 mM Tris-HCl buffer (pH 6.8), 0.1% SDS, 0.136% TEMED and 0.2 ml ammonium persulphate (15 mg/ml) in a total of 7.3 ml. After mixing, the 'stacking' gel solution was gently poured into the gel chamber over the polymerized 'resolving' gel. A teflon 'comb' was then inserted into the stacking gel and the gel allowed to polymerize for at least 2 hours. Tris-Glycine buffer (25 mM Tris-192 mM Glycine) pH 8.3 containing 0.1% SDS was added to both chambers of the electrophoresis cell, followed by the application of 60 μl of the protein samples to the wells created by the teflon 'comb' using a 100 ml Hamilton syringe and the gel run at 25 mA (constant current) for 12 hours. The protein standards used for estimation of molecular weights (in daltons) were; myosin (200,000), β-galactosidase (116,250), phosphorylase (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

b. Acid-Polyacrylamide Gel Electrophoresis: The acyl phosphate intermediate of plasma membrane Ca^{2+}-ATPase was detected by

acid-polyacrylamide (8%) slab gel electrophoresis according to the method of Lichtner and Wolf (1979). The 'resolving' gel consisted of 10.7 ml acrylamide-methylene-bisacrylamide (30%: 0.8%) mixture, 100 mM Tris-phosphate buffer (pH 7.0), 0.2% lithium dodecyl sulphate (LiDS), 0.085% TEMED and 0.8 ml ammonium persulphate (15 mg/ml) in a total of 40 ml. The 'stacking' gel (5%) contained 1.25 ml of acrylamide-methylene-bisacrylamide (30%: 0.8%) mixture, 125 mM Tris-phosphate buffer (pH 6.5), 0.2% LiDS, 0.13% TEMED and 0.3 ml ammonium persulphate (15 mg/ml) in a total of 7.4 ml. Protein samples (60 µl) were run in a 0.2 M Tris-phosphate buffer (pH 7.0) containing 0.2% LiDS at 4°C under the constant current (40 mA) for 7 hours. The protein standards used were similar to those used for the gradient gel studies.

   c. **Staining, destaining and Autoradiography:** The gels were stained with 0.25% Coomassie Brilliant Blue in methanol/acetic acid/water (5:1:4) for 30 min at room temperature, followed by a first destaining procedure in methanol/acetic acid/water (45:9:46) for 1 hour (3 changes) and a second destaining procedure in methanol/acetic acid/water (4:1:15) until the background was transparent.

   Prior to drying, the gels were fixed in acetic acid/glycerol/water (10:3:87) for 30 min, then 70% methanol for 20 min. The gels were immediately dried under vacuum at 80°C for 2 hours. The dried gels were exposed to X-ray film (Kodak Min-R) along with an intensifying screen (Cronex Lightning Plus, DuPont) for 7-16 days at -80°C following which the films were developed.

16. Miscellaneous Methods

a. **Protein Assay**: Pancreatic acinar membrane preparations (10-150 μg protein) were suspended in distilled water to a final volume of 1.5 ml. To this was added 12.5 μl of a 2% deoxycholate solution. Following a ten minute incubation at room temperature, 0.5 ml of cold trichloroacetic acid (24%) was added to precipitate any protein. The suspension was centrifuged at 3000 x g for thirty minutes, the supernatant aspirated, and the pellet resuspended in water and assayed for protein using the standard Lowry (1951) protein assay. Bovine serum albumin was used as the standard protein.

b. **Statistical Analysis**: Students "t" test for unpaired, common variance data was used as a measure of significance. Standard error of the mean (S.E.M.) was used as a measure of variation.

c. **Determination of Free Calcium Concentrations**: Free calcium values were calculated using the ..Fortran program CATIONS (Goldstein, personal communication). The program calculates the apparent association constant of EGTA, EDTA and ATP for calcium and magnesium, adjusted for temperature, pH and ionic strength. Log association constants used were first to fourth proton association with EGTA: 9.235, 8.615, 2.660, 2.000; CaEGTA: 10.630, 5.190: MgEGTA: 5.422, 3.810; HATP: 6.544, 3.945; CaATP: 3.742, 1.890; MgATP: 4.228, 2.240; EDTA: 10.009, 5.987, 2.720, 2.009; CaEDTA: 10.423; MgEDTA: 8.929, 2.770.

RESULTS

1. Determination of Ca^{2+} -ATPase Activity in Pancreatic Plasma Membranes

In preliminary experiments ATPase activity was determined in plasma membranes prepared from whole tissue homogenates. A high ATP hydrolytic activity was observed in the pancreatic plasma membrane preparations. The activity was stimulated by Ca^{2+} in the absence of added Mg^{2+}. Also Mg^{2+} could substitute for Ca^{2+} in activation of the enzyme. Figure 2 shows that the ATPase activity increased with increasing concentrations of Ca^{2+} or Mg^{2+} up to 0.25 mM and levelled off. A combination of saturating concentrations of Ca^{2+} and Mg^{2+} produced the same maximum activity, suggesting that the Ca^{2+} or Mg^{2+} stimulated activities represent the same enzyme. Figure 3 indicates the ATP dependency of the ATPase activity. ATPase activity in the presence of either Ca^{2+} or Mg^{2+} increased with increasing ATP concentration, reaching a maximum at 0.35 mM. Higher concentrations than 0.5 mM were inhibitory.

Since the pancreas is a heterocellular organ, the Ca^{2+}-ATPase activity observed in plasma membranes prepared from pancreatic tissue homogenates could not be directly related to acinar cell function. Characterization of a Ca^{2+}-ATPase activity in pancreatic acinar plasma membranes would require preparation of hormone responsive dissociated pancreatic acinar cells, and identification of possible Ca^{2+}-mediated processes that could be related to amylase secretion in the acinar cell.

Figure 2 Ca^{2+} or Mg^{2+} activation of ATPase activity in pancreatic plasma membrane preparations. ATPase activity was determined as described in Methods at 0.5 mM ATP in the presence of varying concentrations of Ca^{2+} (●——●) or Mg^{2+} (○---○). Results represent the mean ± SEM of five different determinations.

ATPase Activity : μmoles Pi.mg prot.$^{-1}$hr^{-1}

ion conc (mM)
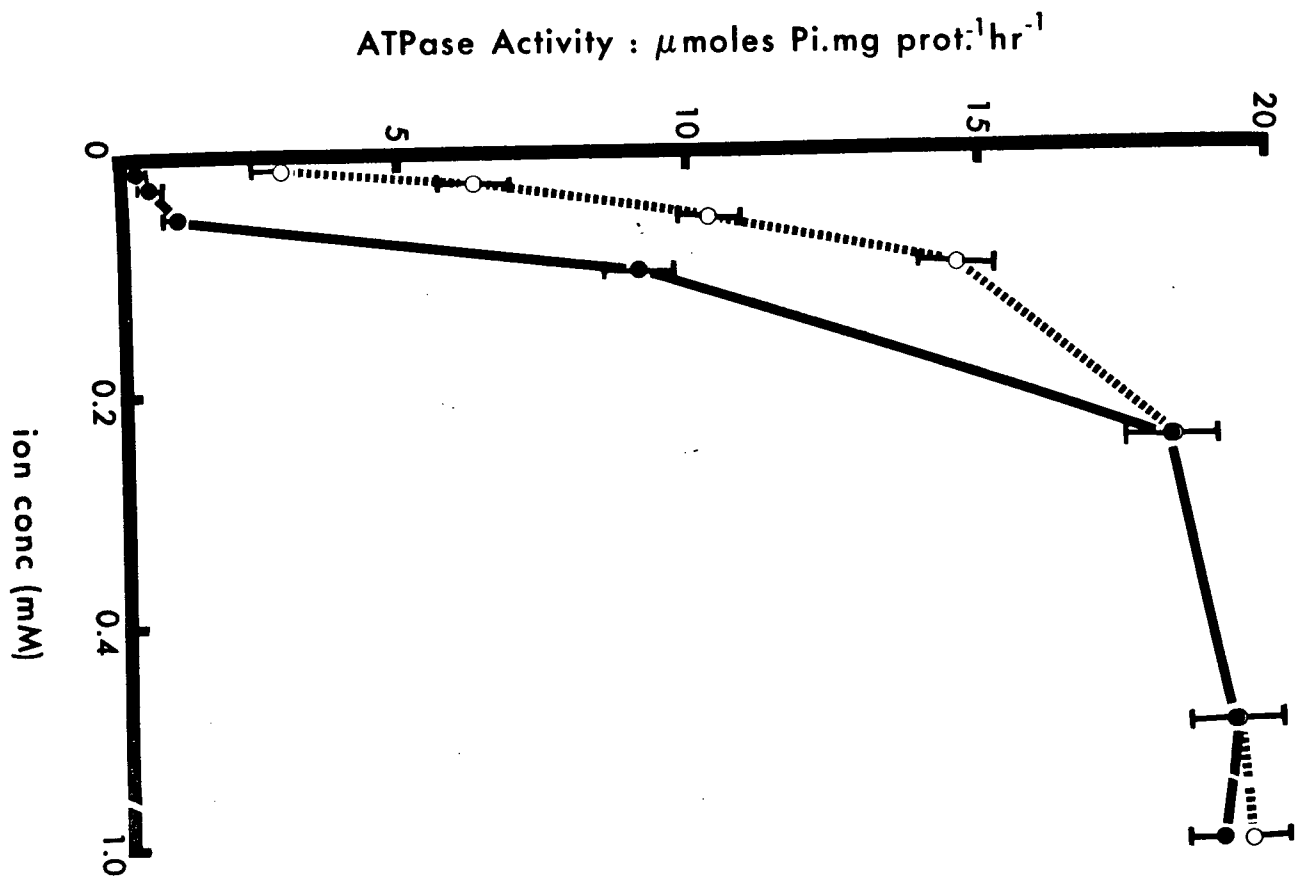
Figure 3 Effect of ATP on ATPase activity in pancreatic plasma
membrane preparations. ATPase activity was determined as
described in Methods at varying concentrations of ATP
(0.01 - 1.0 mM) in the presence of 0.5 mM Ca^{2+} (●——●)
and 0.5 mM Mg^{2+} (○---○). Results represent the mean ±
SEM of five different determinations.
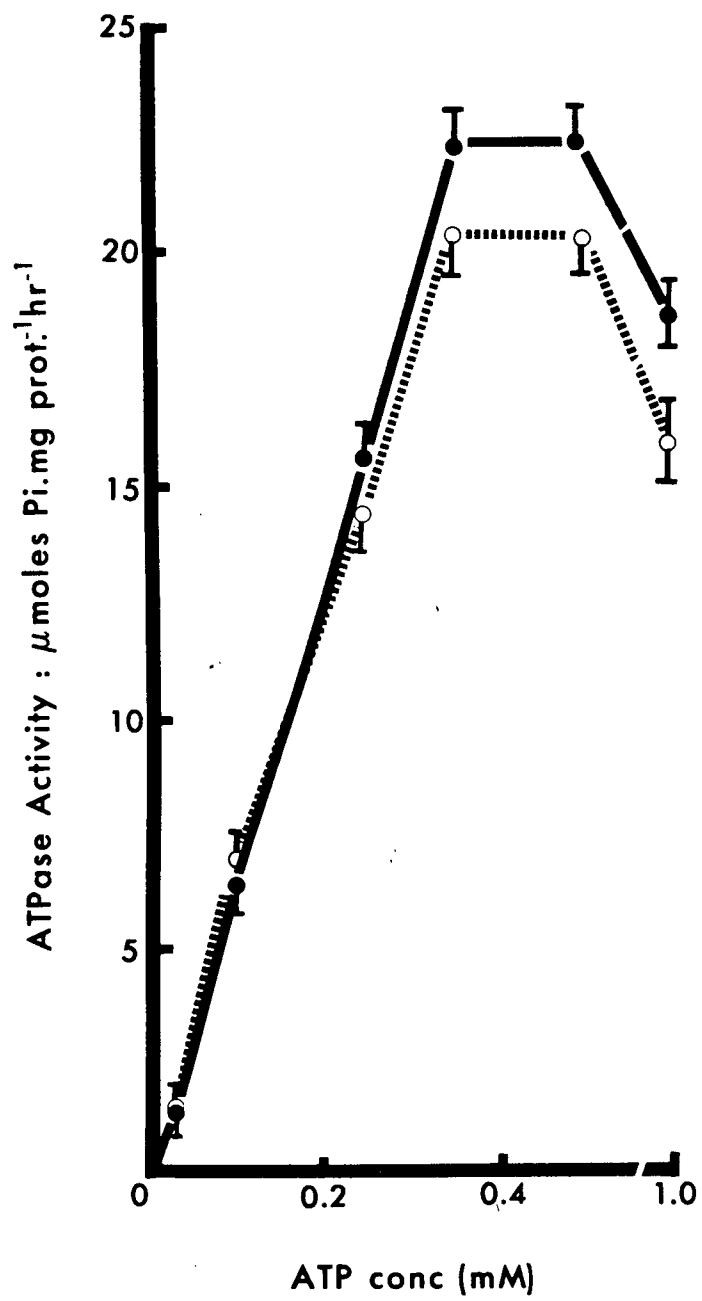
2. Determination of a Possible Relationship of Ca^{2+}-ATPase Activity to Amylase Secretion

a. Characterization of Amylase Secretion in Pancreatic Acinar Cells

The initial objective was to prepare hormone responsive acinar cells from rat pancreas and to determine the purity of the acinar cell population. The first approach was to determine whether the population of cells obtained was enriched with respect to acinar cells. The cellular marker used was the presence of insulin. As shown in Table I there was a 35 - 75% decrease in insulin levels associated with acinar cells compared to the mixed cell population. Since the presence of insulin receptors on acinar cells has been reported (Korc et al 1978), the insulin levels measured in the final preparation might represent insulin bound to the acinar cells, rather than contamination from β-cells. To verify this, cytochemical methods were employed. Figure 4 shows an haematoxylin and eosin staining of the acinar cells. Acinar cells were characterized by their size and granular appearance. The Figure (Figure 4) shows predominantly one cell type with features typical of acinar cells. The majority of the cells maintained their shape and exhibited an acinar configuration. The viability of the cells was greater than 95% as evaluated by the exclusion of trypan blue dye.

To determine the time course of amylase secretion, dissociated rat pancreatic acinar cells were incubated under control conditions and the secretion of amylase in response to 10^{-5} M carbachol was monitored at 15, 30, 45, 60, 75 and 90 min (Figure 5). Amylase secretion increased

Table I

Insulin Content of Enzymatically Dispersed Pancreatic Cell
Homogenates and Isolated Acinar Cells.

	Insulin Content(mU/mg Tissue)		Purification
	Homogenate	Acinar Cells	
Preparation 1	5.66	3.71	1.5 fold
Preparation 2	5.94	1.66	3.5 fold

Insulin was extracted from cells as described in Methods. Insulin
content was assayed using a radioimmunoassay kit and is expressed as
mU/mg of cellular protein.

Figure 4 Light micrograph of isolated pancreatic acinar cells.
Magnification: X 1019.
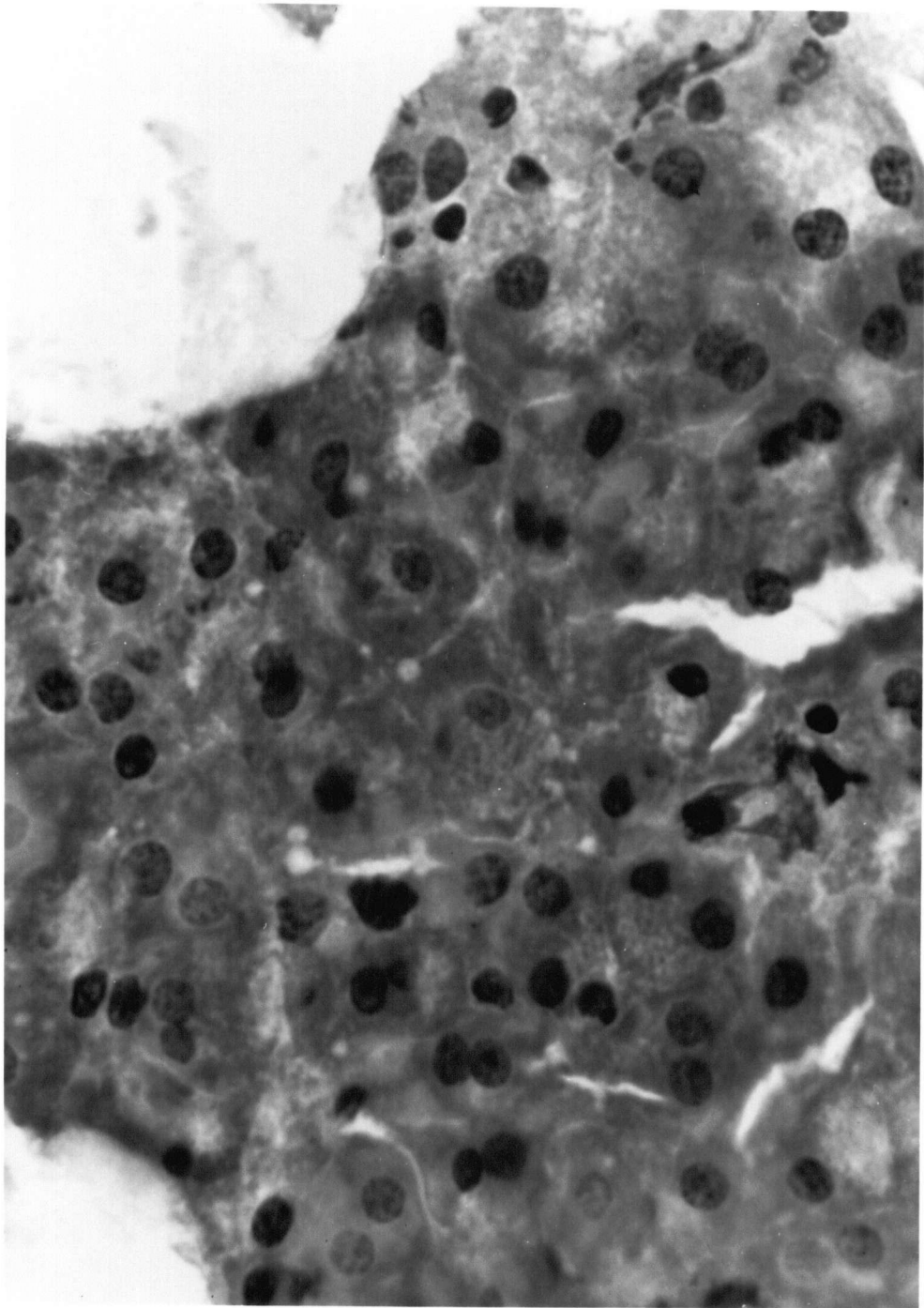
Figure 5 Time course of amylase secretion by dissociated rat
pancreatic acinar cells. Cells were incubated in
standard incubation solution as described in Methods in
the presence (●——●) or absence (○---○) of 10^{-5} M
carbachol. Results shown are the mean ± SEM from three
separate experiments.
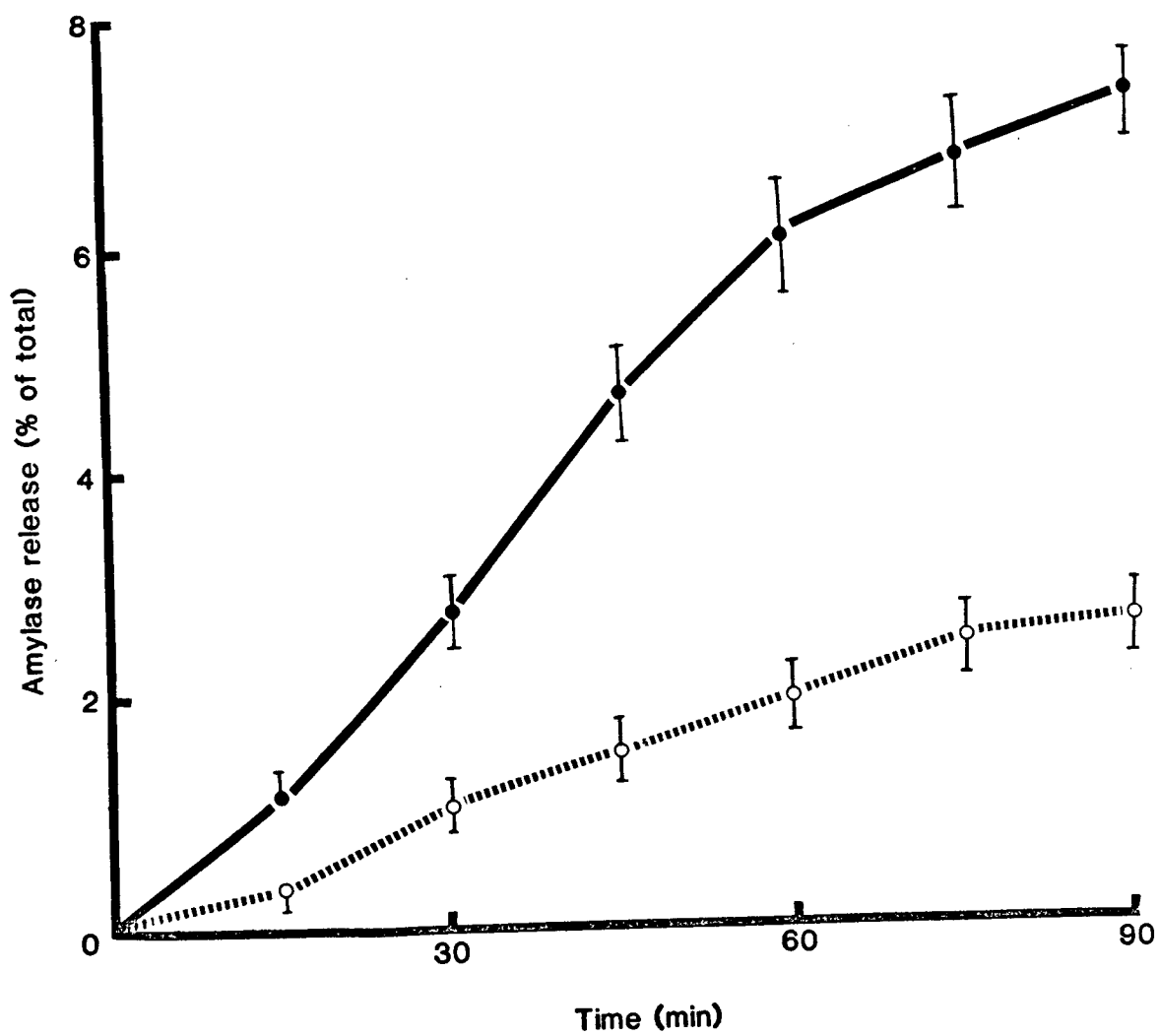
linearly with time up to 60 min, levelling off thereafter. Amylase release increased about three-fold over basal at all time points studied. Figure 6 illustrates the effect of varying concentrations of carbachol, secretin and pancreozymin on amylase release from dissociated pancreatic acinar cells. With increasing concentrations of carbachol and pancreozymin, the rate of amylase secretion increased, becoming maximal at 10^{-5} M and 0.01 U/ml respectively, and then decreased. With secretin as the agonist, amylase release was submaximal at the highest concentration tested (10^{-6} M).

The rate of amylase secretion observed in these studies is comparable to those reported by other workers using similar preparations (Gardner and Jackson 1977; Williams et al 1978). The decreased amylase release at higher carbachol and pancreozymin concentrations has been termed "desensitization" (Williams et al 1978). This is attributed to the fact that these secretagogues produce much higher increases in intracellular Ca^{2+} levels than is required for amylase secretion. The excess Ca^{2+} levels then either directly or indirectly inhibit amylase release.

b. Determination of the Effect of Anticalmodulin Agents on Amylase Secretion

Calmodulin plays a major role in the modulation of a wide variety of Ca^{2+}-dependent cellular functions (Scharff 1981). Based on the reported presence of calmodulin in the pancreas (Vandermeers et al 1977; Bartelt and Scheele 1980), its role in amylase secretion from dissociated rat

Figure 6   Concentration dependence of amylase secretion by
            dissociated rat pancreatic acinar cells. Amylase
            secretion stimulated by secretin (●——●), carbachol
            (○---○) and pancreozymin (▲——▲) was measured in cells
            suspended in standard incubation medium containing 2.5 mM
            Ca^{2+} and 1.2 mM Mg^{2+} at 37°C for 45 min. Results shown
            are mean ± SEM of five different experiments.
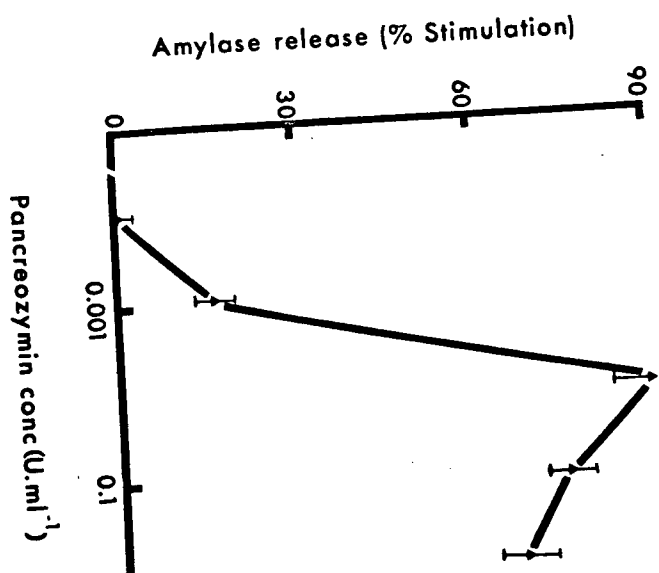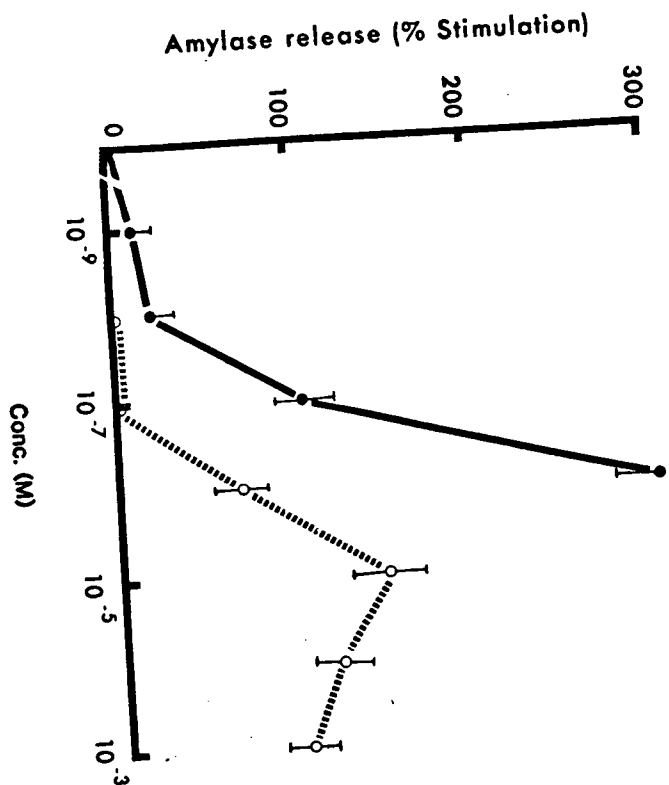
acinar cells was studied indirectly by determining the effects of phenothiazines on the secretory process. These agents are known to bind to calmodulin in a Ca^{2+}-dependent manner and thereby inhibit calmodulin mediated events (Levin and Weiss 1977). As shown in Figure 7, the phenothiazine trifluoperazine (TFP) inhibited carbachol stimulated activity in a dose-dependent manner, with no significant effect on basal amylase release. The concentration of TFP required for 50% inhibition was found to be 10 μM. In Figure 8a the effect of another phenothiazine, chlorpromazine (CPZ) on amylase release induced by various classes of secretagogues was determined. CPZ at a concentration of 10^{-5} M inhibited both carbachol-(10^{-5} M) and pancreozymin-(0.01 U/ml) stimulated amylase release by about 60 and 65% respectively whereas secretin-(10^{-5} M) stimulated release was still virtually intact (98% of maximal response). At higher CPZ concentrations (10^{-4} - 0.5 x 10^{-3} M) all three secretagogues were inhibited to a similar degree. CPZ did not though, have any effect on the basal release of amylase. It appears that at lower concentrations (10^{-6} - 10^{-5} M), CPZ inhibits amylase release by secretagogues (carbachol and pancreozymin) that act by mobilizing intracellular calcium. CPZ at the same concentration has no effect on amylase release induced by secretin, a non-calcium mediated secretagogue. Propranolol, which can produce non-specific membrane stabilizing effects, did not show this selective inhibition (Figure 8b).

These studies indicate the possibility that phenothiazines inhibit calcium mediated amylase release from pancreatic acinar cells by acting on a calmodulin regulated step in the stimulus-secretion coupling

Figure 7   Effect of trifluoperazine on amylase secretion from
            dissociated pancreatic acinar cells. Enzyme secretion
            was measured in the presence (●━●) and absence (○---○)
            of 10^{-5} M carbachol. Results represent the mean ±S.E.M.
            of five separate experiments.
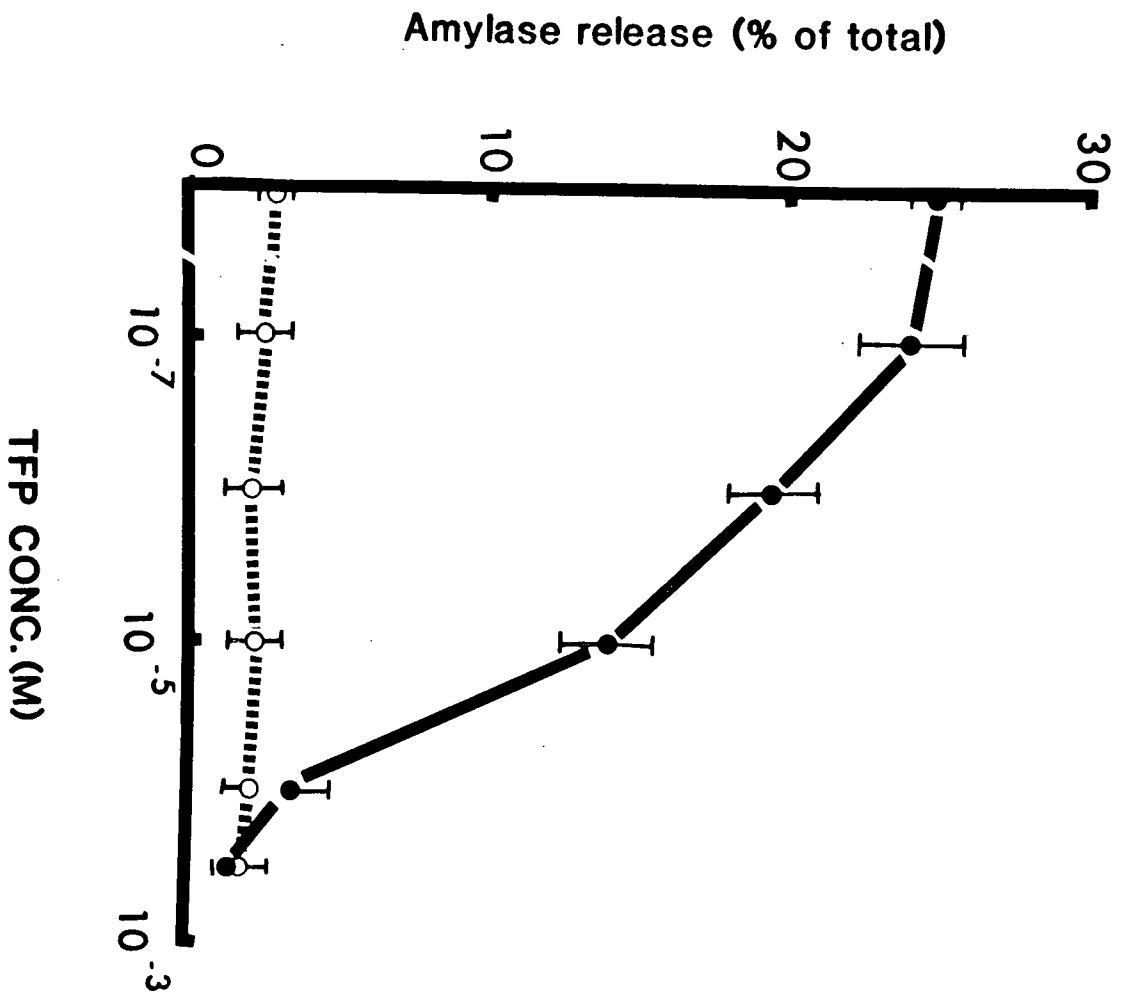
Figure 8a   Effect of chlorpromazine on amylase secretion by
             dissociated pancreatic acinar cells. Amylase secretion
             was measured in the presence of 10^{-5} M carbachol (●—●).
             0.01 U/ml pancreozymin (▲——▲) and 10^{-6} M secretin
             (○---○). Cells were suspended in standard incubation
             medium containing 2.5 mM Ca^{2+} and 1.2 mM Mg^{2+} at 37°C
             for 45 min. Results represent the mean ± SEM of three
             separate experiments.

Figure 8b   Effect of propranolol on amylase secretion by dissociated
             pancreatic acinar cells. Amylase secretion was measured
             in the presence of 10^{-5}M carbachol (●—●) and 10^{-6}M
             secretin (○---○). Cells were suspended in standard
             incubation solution containing 2.5 mM Ca^{2+} and 1.2 mM
             Mg^{2+} at 37°C for 45 min. Results represent the mean
             ±SEM of three separate experiments.
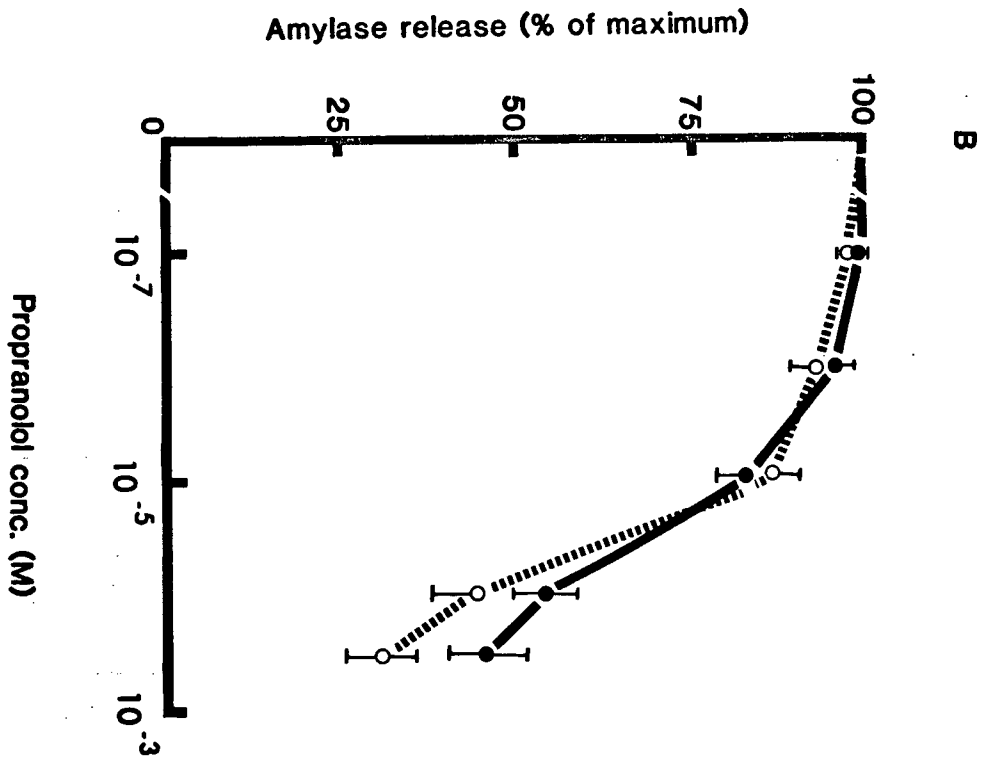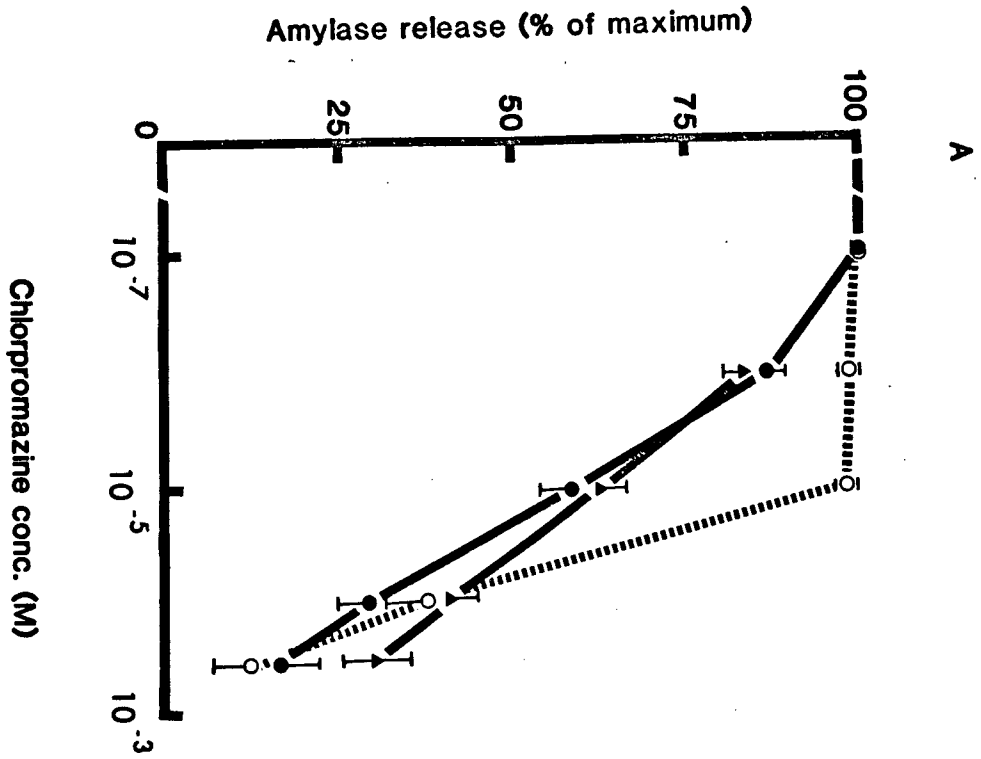
process. The mechanism by which calmodulin would regulate pancreatic

enzyme secretion is not known. A calmodulin-dependent enzyme that may

play a role in the regulation of free intracellular calcium is the

plasma membrane Ca^{2+}-ATPase. Therefore subsequent studies involved

characterization of a Ca^{2+}-ATPase activity in plasma membranes obtained

from pancreatic acinar cells.

3. Characterization of Ca^{2+}-ATPase Activity in Pancreatic Acinar Plasma Membranes

Membrane preparations obtained from whole tissue homogenates could

be contaminated with plasma membranes of cells from the endocrine

pancreas, the collecting duct and some centroacinar cells. Therefore

attempts were made to prepare plasma membranes from acinar cells, the

cells mainly concerned with secretion of digestive enzymes. The

procedure first involved the isolation of acinar cells as described in

Methods and then the preparation of plasma membranes from these cells.

The acinar cells were viable and responsive to stimulation by a number

of agents (see above). This procedure yielded pure acinar plasma

membranes without appreciable loss of endogenous ATPase activity.

Table II illustrates the sub-cellular distribution of marker enzymes

among various fractions obtained from acinar cell homogenates. The

fraction collected from the 27%/35% sucrose interface was enriched in

enzymes characteristic of plasma membranes. There was a 12-fold

increase in $(Na^+ + K^+)$-ATPase, 4-fold increase in 5'-nucleotidase and

Table II

Specific Activity of Ca^{2+}-ATPase and Marker Enzymes
in Cell Homogenates and Subcellular Fractions.

	$(Na^+ + K^+)$-ATPase[a]	5'-Nucleotidase[a]	Cytochrome c[b] Oxidase	Ca^{2+}-ATPase[a,c]
Homogenate	0.64 ± 0.05	1.52 ± 0.09	4.51 ± 0.15	0.18 ± 0.02
10-25% sucrose interface	3.62 ± 0.31	2.45 ± 0.16	0.34 ± 0.08	0.32 ± 0.01
27-35% sucrose interface	7.89 ± 0.12	5.51 ± 0.10	0.67 ± 0.05	0.80 ± 0.03
Bottom pellet	0.31 ± 0.03	0.26 ± 0.06	6.57 ± 0.27	0.05 ± 0.01

[a] μmoles Pi/mg prot/hr.

[b] nmoles cyt c oxidized/mg prot/min.

[c] Ca^{2+} concentration is 0.4 μM free.

4-fold increase in Ca^{2+} stimulated ATPase activities. Cytochrome c oxidase activity was significantly decreased indicating that mitochondrial contamination was minimal in this fraction. The differential degrees of purification of marker enzymes for plasma membranes may be due to the different stabilities of the enzymes studied.

A high ATP hydrolytic activity was observed in the plasma membrane enriched fraction. As shown in Figure 9, the ATPase activity increased with increasing concentrations of Ca^{2+} or Mg^{2+} up to a maximum of 88 μM free ion concentration. Kinetic analysis indicated that the system has a significantly higher affinity for Ca^{2+} than Mg^{2+} (P < 0.05) with a similar maximum rate of activity (Table III). The ATPase activity was also activated by Mn^{2+} and Zn^{2+} with Sr^{2+} and Ba^{2+} showing the least activity (Table IV). A combination of saturating concentrations of Ca^{2+} and Mg^{2+} produced the same maximum activity (Table IV). Monovalent cations though, did not affect the activity of the enzyme (Table V). Similarly, carbachol and pancreozymin at concentrations that produce maximal amylase release from acinar cells had no effect on the activity of the enzyme (Table V). Whereas 10 μM CPZ was without effect, 1 mM markedly inhibited the enzyme activity, possibly due to membrane disruption (Table V). The Ca^{2+}-ATPase activity was insensitive to 5 mM sodium azide, indicating that this enzyme is different from the mitochondrial ATPase. A comparison of the substrate requirement for the phosphohydrolase activity is shown in Figures 10 and 11. The profile for the substrate requirement is similar for both Ca^{2+} and Mg^{2+}

Figure 9 The effect of Mg^{2+} and Ca^{2+} on ATPase activity in
plasma-membrane enriched fractions of pancreatic acinar
cells.
ATP hydrolytic activity in the presence of increasing
Ca^{2+} (●──●) and Mg^{2+} (O---O) concentrations. Results
represent the mean ± S.E.M. of five different
determinations.

ATPase Activity : μmoles Pi.mg prot^{-1}hr^{-1}

$-$Log [Ca^{2+}] or [Mg^{2+}] (M)
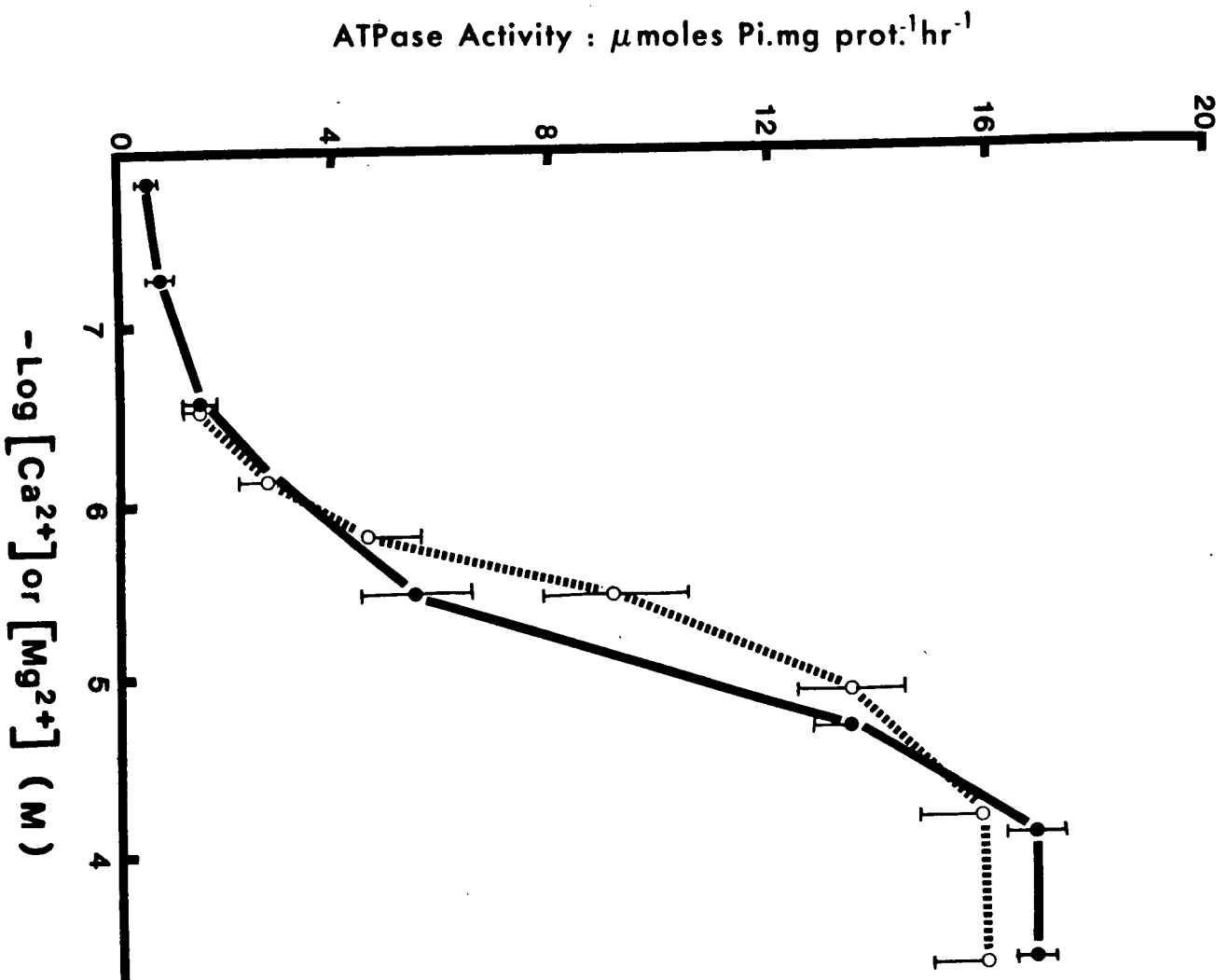
Table III

K_{diss} and V_{max} of the ATPase Activity of Pancreatic
Acinar Plasma Membrane Preparations in the Presence of
Ca^{2+} or Mg^{2+}.

	K_{diss} (μM)	V_{max} (μmoles Pi/mg prot/hr)
Ca^{2+} (5)[a]	1.73 ± 0.25[b,c]	15.29 ± 0.76
Mg^{2+} (5)	2.98 ± 0.39	16.32 ± 0.61

[a]Number of preparations.

[b]Mean $\pm$ SEM in each case.

[c]Significant ($p < 0.05$) when compared to Mg^{2+}-stimulated activity.

Table IV

ATPase Activity of Pancreatic Acinar Plasma Membrane
Preparations in the Presence of Divalent Cations.

Cation	Specific Activity (μmoles Pi/mg prot/hr)
Ca^{2+}	13.42 ± 0.69
Mg^{2+}	13.45 ± 0.99
Mn^{2+}	10.32 ± 0.46
Zn^{2+}	8.69 ± 0.32
Sr^{2+}	1.40 ± 0.05
Ba^{2+}	1.02 ± 0.07
$Ca^{2+} + Mg^{2+}$	13.56 ± 0.08

ATPase activity was measured in a medium containing 48 mM Tris-HCl pH
7.4, 0.5 mM ATP, 0.1 mM ouabain, 2 mM NaN_3 and 0.25 mM divalent cation
at 37°C. Results represent the mean ± S.E.M. of four separate
determinations.

Table V

Effect of Monovalent Cations and Drugs on the ATPase Activity of
Pancreatic Acinar Plasma Membrane Preparations.

| | ATPase Activity (μmoles Pi/mg prot/hr) | |
	Ca^{2+}	Mg^{2+}
Control	16.01 ± 1.5	16.03 ± 1.2
100 mM Na^+	15.01 ± 1.3	14.85 ± 1.1
100 mM K^+	13.72 ± 0.9	14.78 ± 0.9
10^{-5} M Carbachol	15.73 ± 2.3	15.59 ± 1.4
10^{-5} M Chlorpromazine	14.04 ± 1.2	16.59 ± 2.0
10^{-3} M Chlorpromazine	3.46 ± 0.2	1.95 ± 0.5
0.01 U/ml Pancreozymin	16.92 ± 0.2	16.66 ± 1.0
5 mM sodium azide	15.23 ± 1.5	15.01 ± 1.7

ATPase activity was measured in a medium containing 48 mM Tris-HCl pH
7.4, 0.06 mM EGTA, 1 mM ouabain, 2 mM NaN_3 and 0.5 mM ATP at 37°C. When
either Ca^{2+} or Mg^{2+} was added, it was present at a concentration of 88
μM free. Results represent the mean ± S.E.M. of five different
experiments.

Figure 10   Substrate requirement of Ca^{2+}-ATPase activity in plasma
membrane preparations of acinar cells. Enzyme activity
was determined as described in Methods at varying
concentrations (0.01 - 1.0 mM) of ATP (●——●), GTP
(▲——▲), ADP (O---O) and AMP (Δ---Δ) in the presence of
88 μM Ca^{2+}. The results represent the mean ± SEM of five
different determinations.
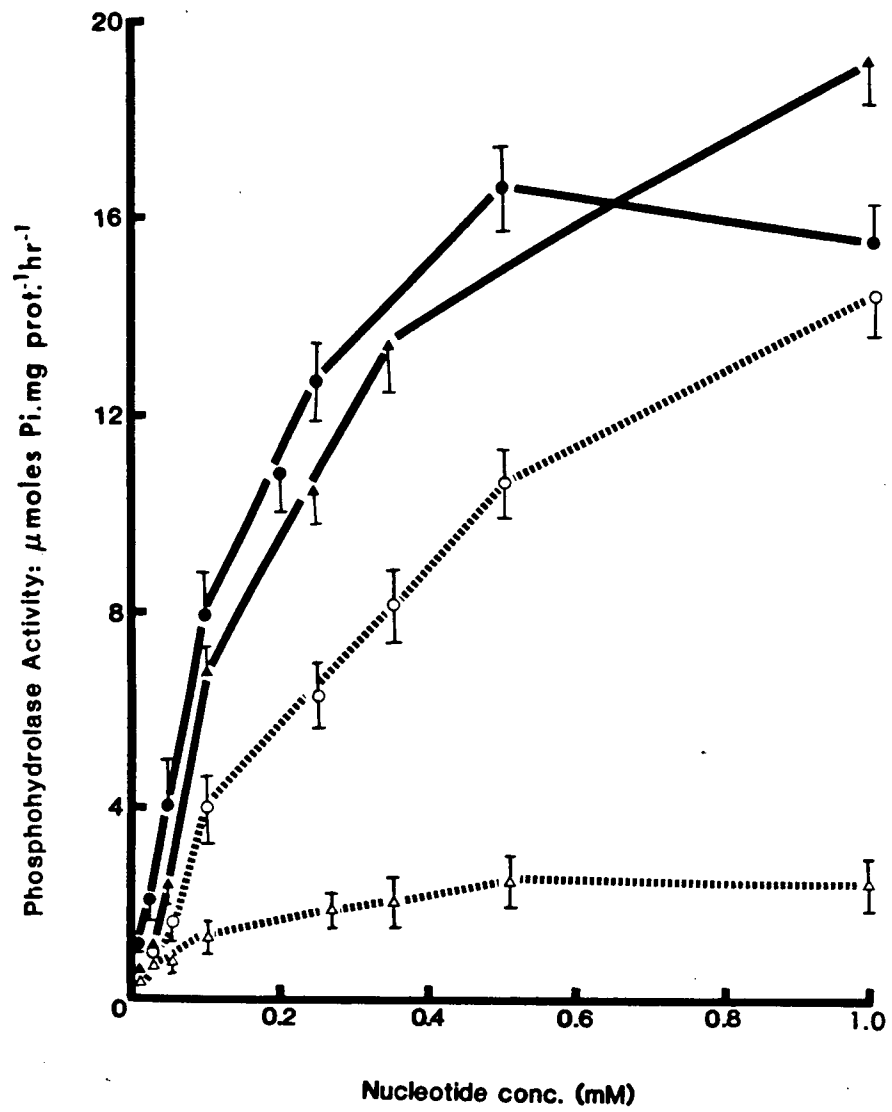
Figure 11   Substrate requirement of Mg^{2+}-ATPase activity in plasma
            membrane preparations of acinar cells. Enzyme activity
            was determined as described in Methods at varying
            concentrations (0.01 - 1.0 mM) of ATP (●——●), GTP
            (▲——▲), ADP (○---○) and AMP (△---△) in the presence of
            88 μM Mg^{2+}. The results represent the mean ± SEM of five
            different determinations.
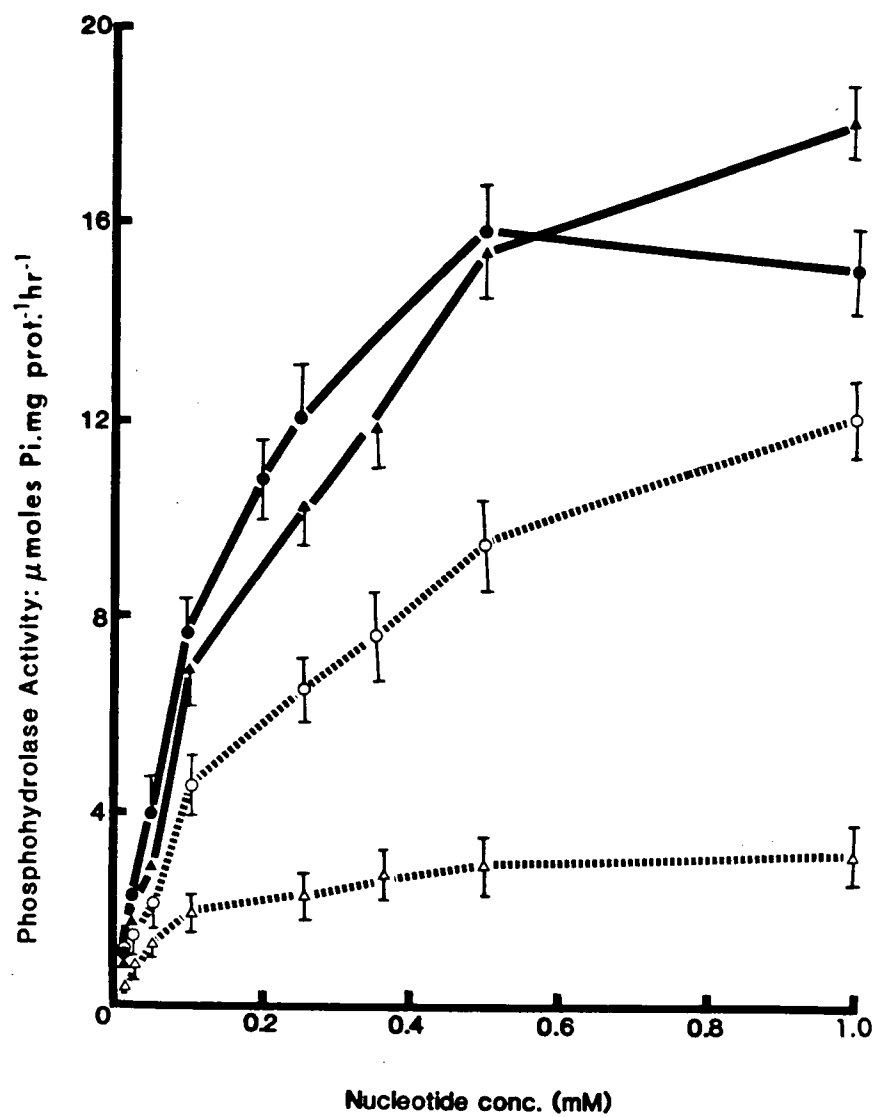
stimulated activities. ATP and GTP were the most suitable substrates, followed by ADP. AMP showed minimal activity possibly due to the presence of 5'-Nucleotidase. Pyrrophosphate could not be used as a substrate, suggesting that the enzyme is not a non-specific phosphatase. The possibility existed that the effect of ADP was due to ATP produced by conversion of ADP by adenylate kinase present in the membrane preparation. To demonstrate that this was not the case, ADPase activity was determined in the presence of the adenylate kinase inhibitor, adenyl 3',5'-Adenosine (Ap5A). As shown in Figure 12, Ap5A at concentrations that generally inhibit adenylate kinase activity in various systems (Feldhaus et al 1975), did not inhibit ADPase activity. Rather, stimulation of the ADPase activity was observed at 0.5 and 1 mM Ap5A. Figure 13 illustrates the effect of pH on the enzyme activity. Ca^{2+} or Mg^{2+} stimulated ATPase activities exhibited a similar pH dependency with a relatively broad pH optimum around pH 8. Figure 14 is an Arrhenius plot of data obtained on the effect of temperature on the activity of the enzyme, indicating a transition temperature of 25°C for Ca^{2+}-ATPase activity and 20°C for Mg^{2+}-ATPase activity.

4. **Regulation of Pancreatic Acinar Plasma Membrane Ca^{2+}-ATPase by Calmodulin**

Having demonstrated that the phenothiazines inhibit amylase secretion in a Ca^{2+}-dependent manner, possibly through calmodulin inhibition, it was of interest to determine whether the Ca^{2+}-ATPase is also regulated by calmodulin. In these experiments low Ca^{2+}

Figure 12   Effect of adenyl 3',5'-adenosine (Ap5A) on Ca^{2+}-ATPase
activity in plasma membrane preparations of acinar
cells. Ca^{2+}-ATPase activity was determined at 88 μM Ca^{2+}
and 0.5 mM ADP. Data represent the mean ±SEM of three
separate experiments.

Ca^{2+}-ATPase Activity: μmoles Pi/mg prot/hr

Ap5A Conc. mM
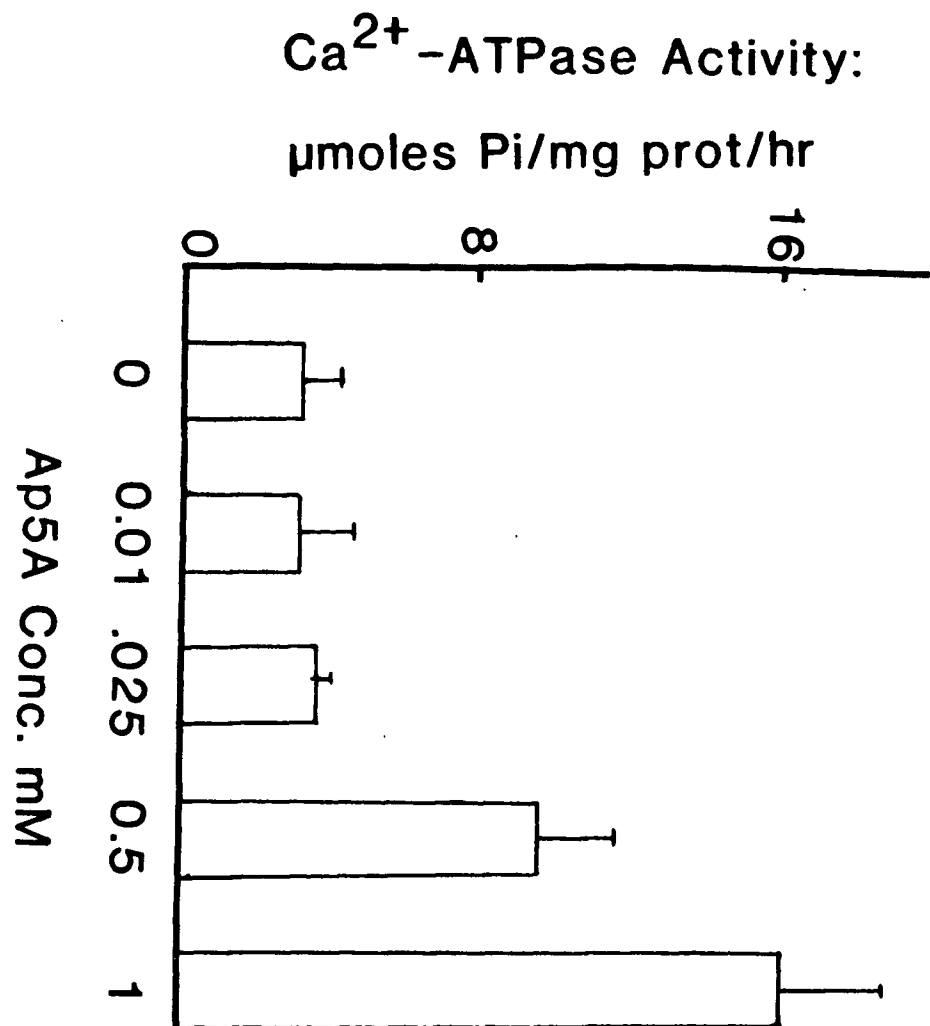
Figure 13 Effect of pH on ATP hydrolytic activity in plasma
membranes of acinar cells. ATPase activity was
determined at 0.5 mM ATP and 88 μM Ca^{2+} (●——●) or 88 μM
Mg^{2+} (○---○). The reaction medium was buffered with
Tris-glycine-maleic acid and adjusted between pH 6 and
9. Results represent the mean of two different
determinations each done in duplicate.

ATPase Activity : μmoles Pi.mg prot.$^{-1}$hr^{-1}

pH

- 96 -
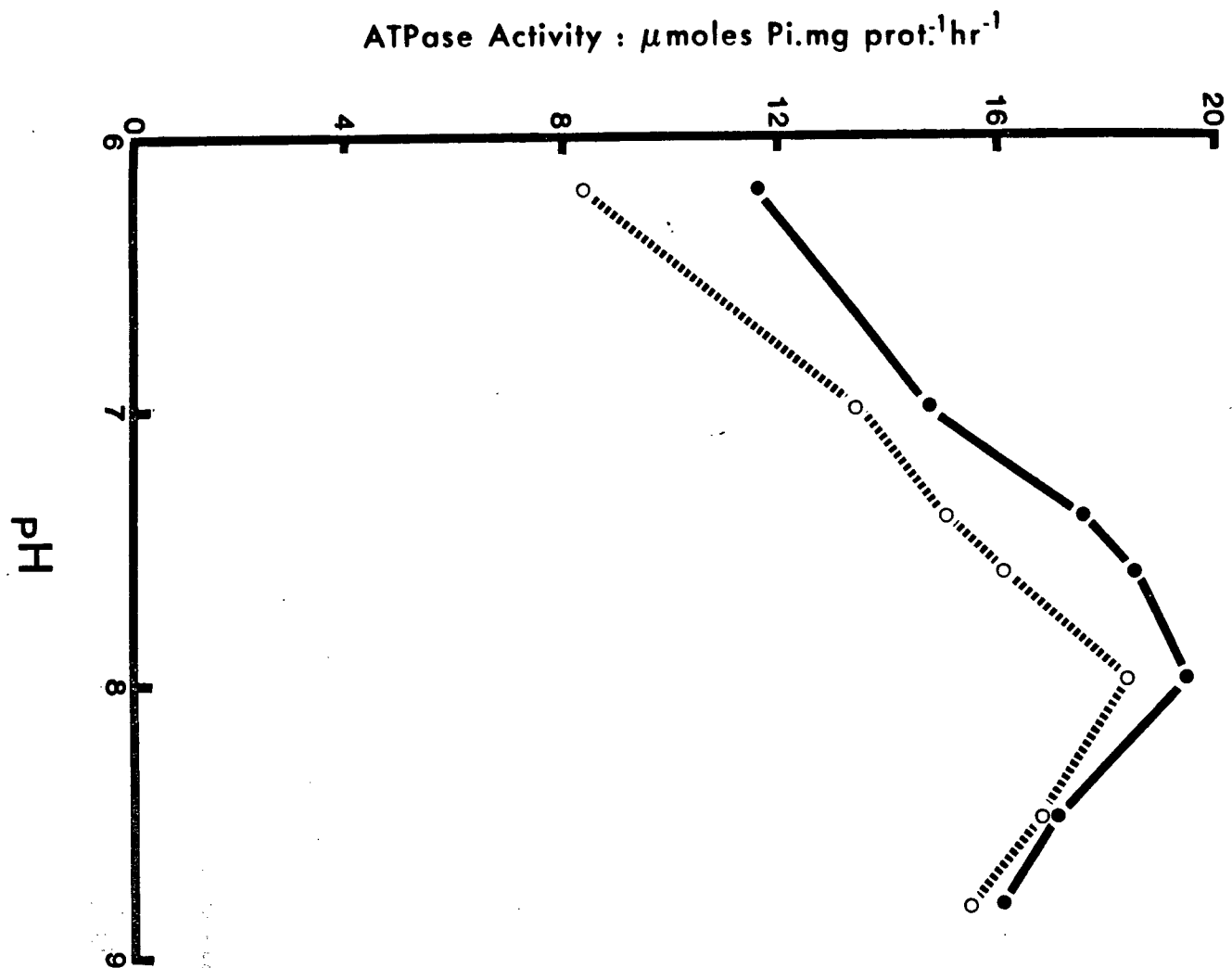
Figure 14   Arrhenius plots of ATPase activity as a function of
            temperature. ATPase activity was determined in acinar
            membrane preparations as described in Methods in the
            presence of 88 μM Ca^{2+} (●——●) or 88 μM Mg^{2+} (o....o).
            The pH was adjusted to pH 7.4 at each temperature
            setting. Results represent the mean of two different
            experiments each done in duplicate.
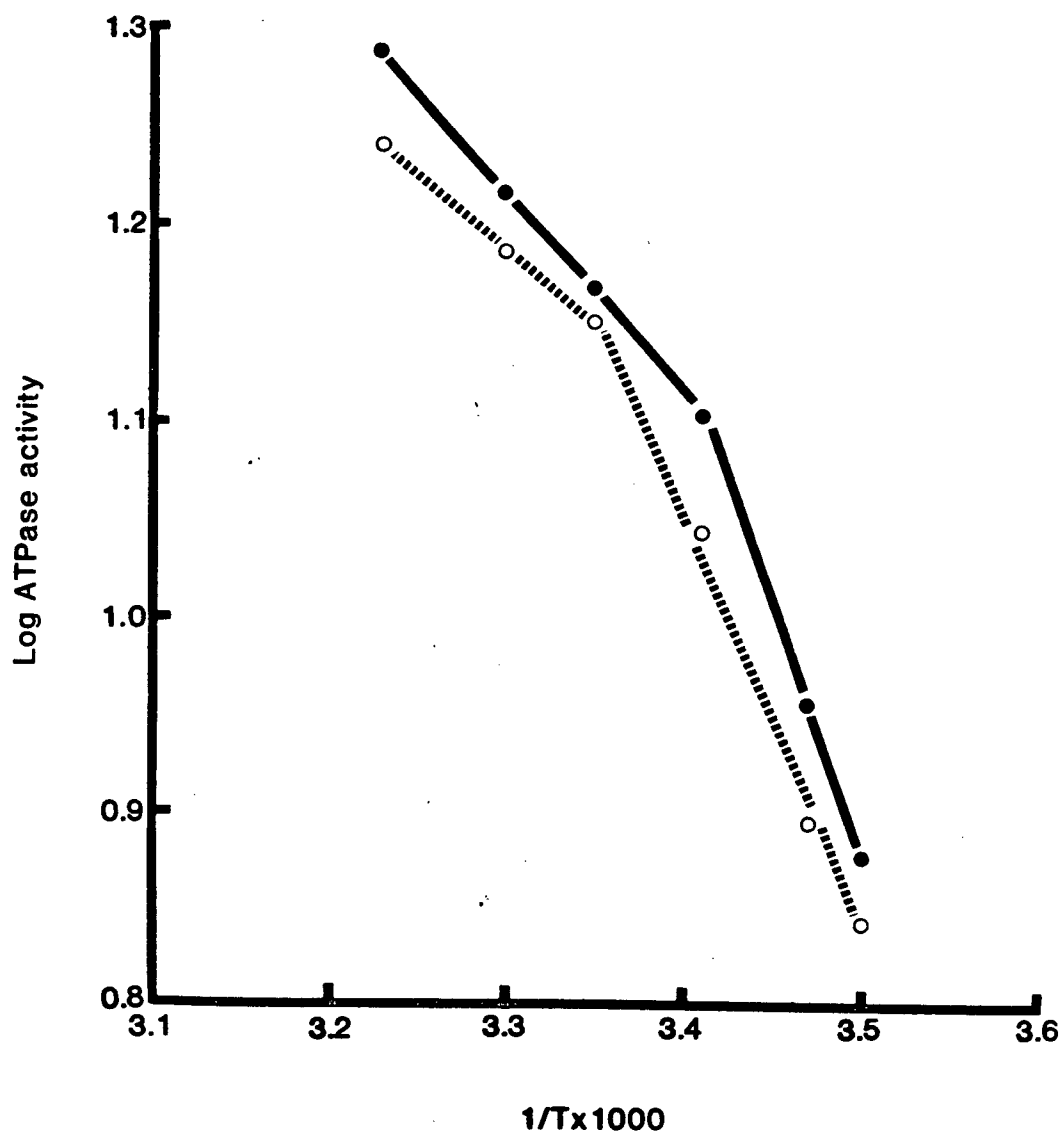
concentrations were used as calmodulin activation of the Ca^{2+}-ATPase was found to be only marginal at higher Ca^{2+} concentrations. In the presence of endogenous Mg^{2+} (1.5 - 2 μM) calmodulin activation of the Ca^{2+} stimulated activity was observed. As shown in Figure 15, exogenous calmodulin stimulated the Ca^{2+}-ATPase activity in a dose-dependent manner. At 280 nM calmodulin, the enzyme activity was increased by 244%, increasing to 890% at 1 μM and showed saturation beyond 1.7 μM. Using a radioimmunoassay technique, the calmodulin content of the plasma membrane preparations was found to be 41.8 ± 3.5 ng/mg protein. The presence of bound calmodulin could in part explain the poor response of the enzyme to exogenously added calmodulin. It was determined if the 'anticalmodulin' agents TFP and CPZ would block calmodulin stimulation of the Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity was measured at 0.4 μM free Ca^{2+} and in the presence and absence of 280 nM calmodulin. Figure 16 shows that while TFP and CPZ (up to 175 μM) did not affect the basal Ca^{2+}-ATPase activity, the phenothiazines inhibited calmodulin stimulated Ca^{2+}-ATPase activity in a dose-dependent manner. The half maximal inhibition of the calmodulin stimulated activity occurred at 30 μM TFP and 55 μM CPZ, with maximal inhibition observed at 125 μM. It was reported by Gietzen et al (1983) that Compound 48/80, unrelated to the phenothiazines, is a more specific inhibitor of calmodulin stimulated Ca^{2+}-ATPases. As shown in Figure 17, compound 48/80 inhibited activation of acinar plasma membrane Ca^{2+}-ATPase activity by calmodulin (200 nM) in a dose-dependent fashion. Compound 48/80, up to 400 μg/ml, did not have any effect on the basal activity.

Figure 15   Concentrations dependence of calmodulin stimulation of
            Ca^{2+}-ATPase activity.   ATPase activity in the presence of
            0.4 μM Ca^{2+} and endogenous Mg^{2+} (measured to be 1-2μM).
            Data represent the mean ±S.E.M. of three separate
            determinations.
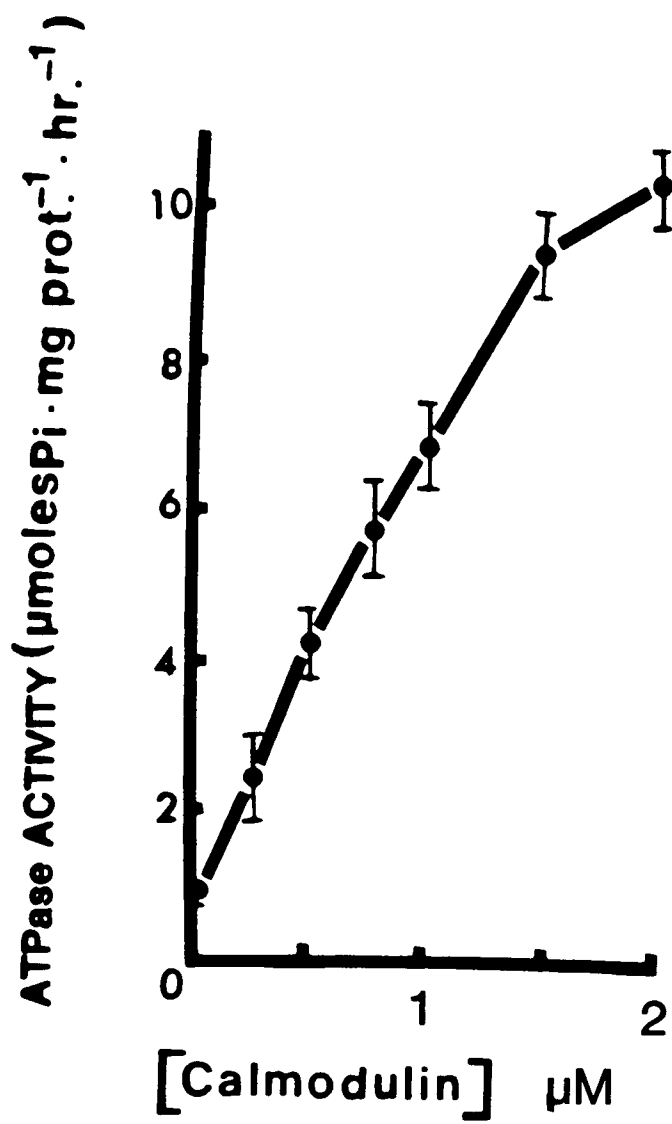
Figure 16   Effect of phenothiazines on calmodulin stimulation of
            Ca^{2+}-ATPase activity in plasma membranes of acinar
            cells. Reaction medium contained 0.4 µM Ca^{2+}, endogenous
            Mg^{2+} (1-2 µM) and trifluoperazine (Δ---Δ) chlorpromazine
            (o....o) or 280 nM calmodulin, trifluoperazine (▲——▲)
            and chlorpromazine (●——●). Data represent the mean ±
            S.E.M. of four separate determinations.

ATPase ACTIVITY (%)

[PHENOTHIAZINE] (μM)
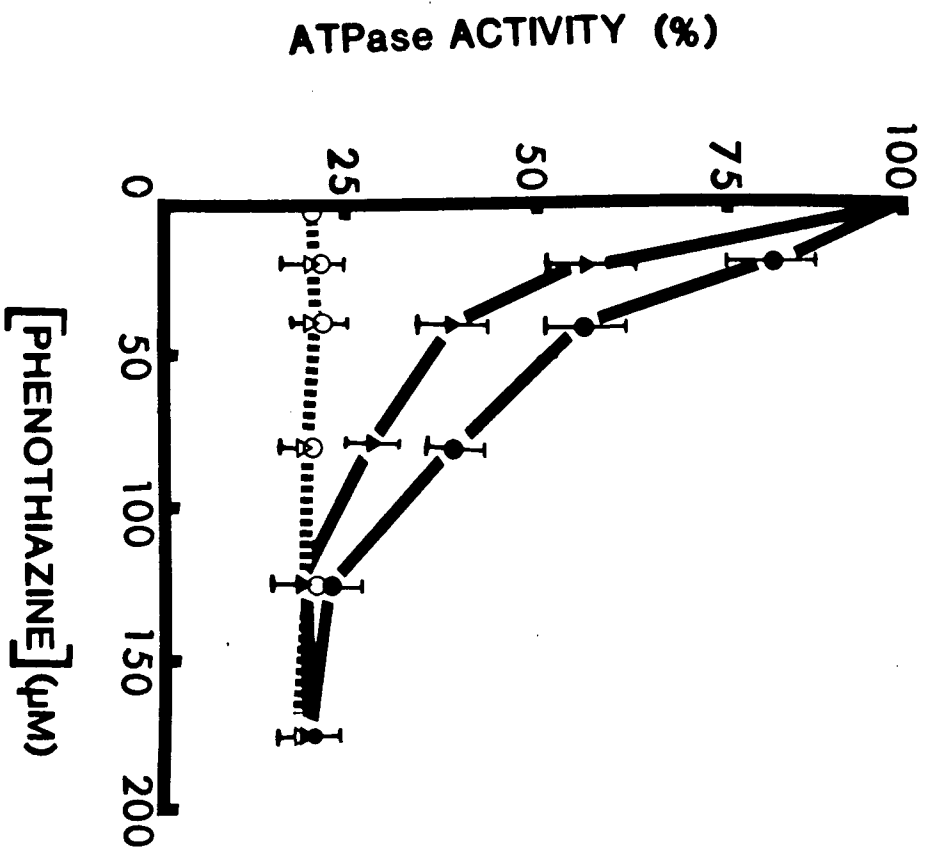
Figure 17 Effect of Compound 48/80 on calmodulin stimulation of
Ca^{2+}-ATPase activity in plasma membranes of acinar
cells. ATPase activity was determined at 0.4 μM Ca^{2+},
endogenous Mg^{2+} (1-2 μM) and in the presence (●—●) and
absence (○—○) of 280 nM calmodulin. Data represent the
mean of two experiments each done in duplicates.
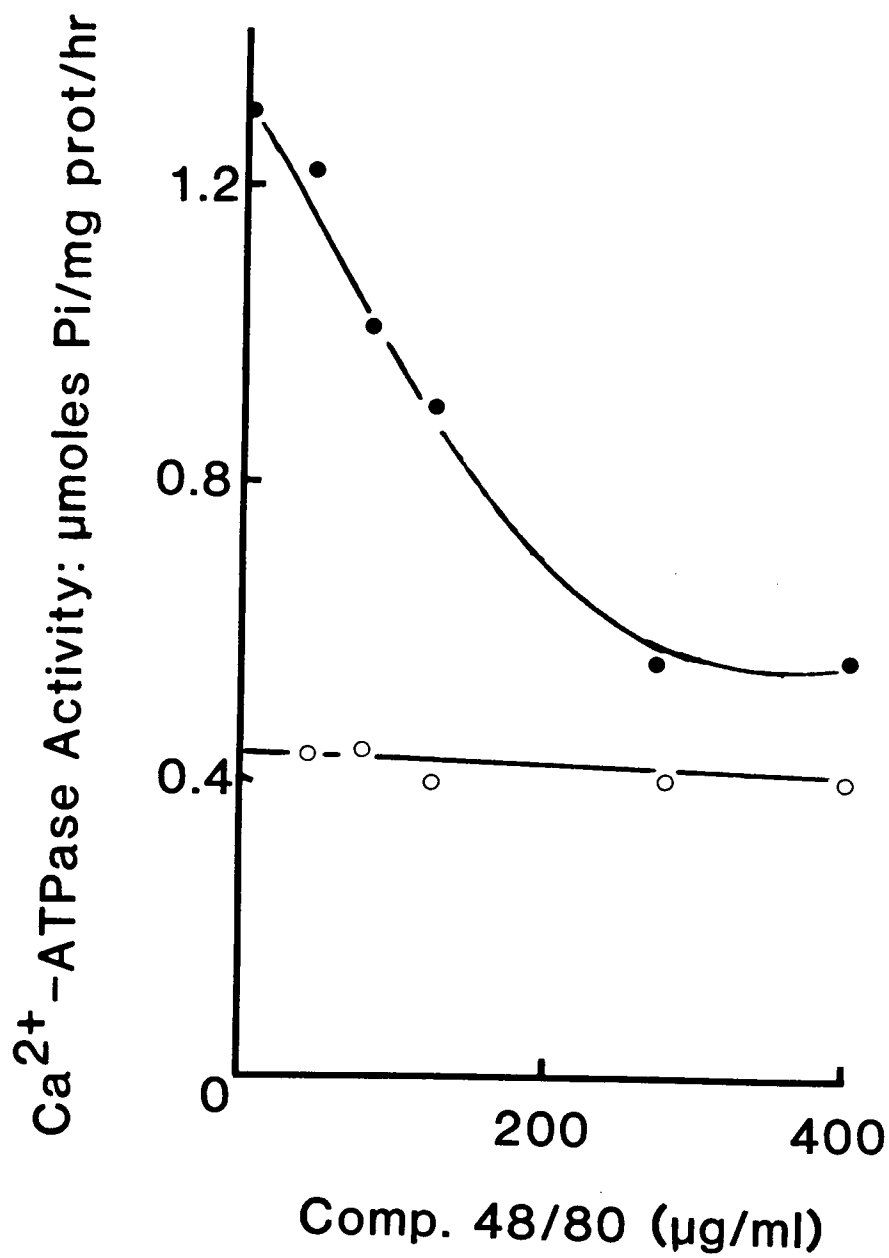
The effect of calmodulin on the Ca^{2+} activation curve is shown in Figure 18. Calmodulin increased the sensitivity of the enzyme system for calcium; no significant effect was observed at higher Ca^{2+} concentrations (>0.4 μM). Calmodulin stimulation was observed in the presence of endogenous calcium, determined by atomic absorption spectrometry to be in the range of 6-7 μM. Maximal calmodulin stimulation occurred at 0.03 μM free Ca^{2+}, with the degree of stimulation decreasing with increasing free Ca^{2+} concentrations. In the presence of endogenous Ca^{2+}, calmodulin stimulation of the Ca^{2+}-ATPase activity was decreased by 20% when 1 mM EGTA was added to the reaction medium (Table VI). In the presence of 1 mM EDTA, calmodulin stimulation was abolished. This could suggest that either calmodulin stimulation of the Ca^{2+}-ATPase activity is dependent on endogenous Mg^{2+} (1.5 - 2 μM total), or that endogenous Mg^{2+} is required for the expression of the basal Ca^{2+}-ATPase activity.

In order to further establish the role of Mg^{2+} in this system, plasma membranes were incubated with 1 mM EDTA at 37°C for 30 min to produce membranes depleted of divalent cations (EDTA-treated membranes). Figure 19 illustrates the effect of exogenously added Mg^{2+} on $(Ca^{2+} + Mg^{2+})$-ATPase activity in EDTA-treated membranes. $(Ca^{2+} + Mg^{2+})$-ATPase activity refers to that activity present following subtraction of the activity measured in the absence of added Ca^{2+} and in the presence of Mg^{2+}. While 0.05 μM free Ca^{2+} failed to stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase activity of EDTA-treated membrane preparations, addition of 0.5 μM Mg^{2+} resulted in stimulation of the ATPase activity. This activity was further increased by 1, 2, and 5 μM Mg^{2+}. The

Figure 18 The effect of exogenous calmodulin on Ca^{2+}-ATPase
activity in plasma membrane enriched preparations of
pancreatic acinar cells. ATPase activity in the presence
of increasing Ca^{2+}-concentrations (o....o) (Mg^{2+}
estimated to be 1-2 μM), and a calmodulin concentration
(CaM) of 280 nM (●━━●) Data represent mean ±SEM of four
different experiments.

ATPase ACTIVITY (μmoles Pi·mg prot.$^{-1}$·hr.$^{-1}$)

−Log [Ca^{2+}] (M)
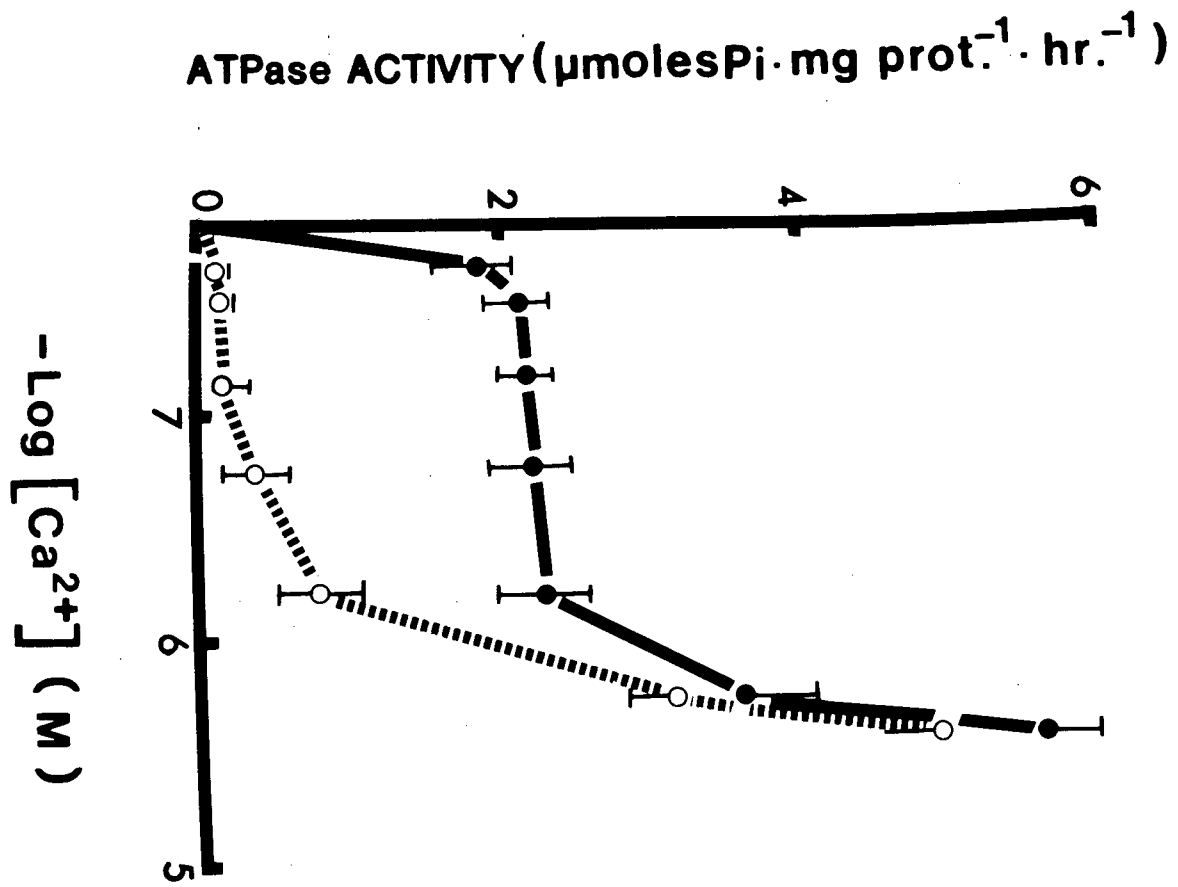
Table VI

Effect of EGTA and EDTA on Calmodulin Stimulated
Ca^{2+}-ATPase Activity.

Addition	Ca^{2+}-ATPase Activity (% Control)
Control[a]	100
Calmodulin	1438 ± 70
Calmodulin + 1 mM EDTA	100 ± 10
Calmodulin + 1 mM EGTA	1154 ± 50

ATPase activity was assayed in 48 mM Tris-HCl pH 7.4, containing 0.06 mM
EGTA, 0.1 mM ouabain, 2 mM NaN_3, 0.5 mM ATP and endogenous Ca^{2+}
(estimated to be 7 μM total). When calmodulin was present it was added
at a concentration of 280 nM. Data represent mean ± S.E.M. of five
different determinations.

[a]Endogenous free calcium concentration is 9.4 nM.

Figure 19 $(Ca^{2+} + Mg^{2+})$-ATPase activity in EDTA treated plasma membranes of acinar cells. Calcium activation curve determined in the absence of $Mg^{2+}(0)$ and in the presence of 0.5 μM (●), 1 μM (Δ), 2 μM (▲) and 5 μM (■) Mg^{2+}. $(Ca^{2+} + Mg^{2+})$-ATPase activity refers to that activity present following subtraction of the activity measured in the absence of added calcium and in the presence of Mg^{2+}. Data represent the mean ±S.E.M. of three separate determinations.

$(Ca^{2+} + Mg^{2+})$–ATPase ACTIVITY

$(\mu moles\, P_i \cdot mg\, prot.^{-1} \cdot hr.^{-1})$

$-Log\,[Ca^{2+}]\,(M)$
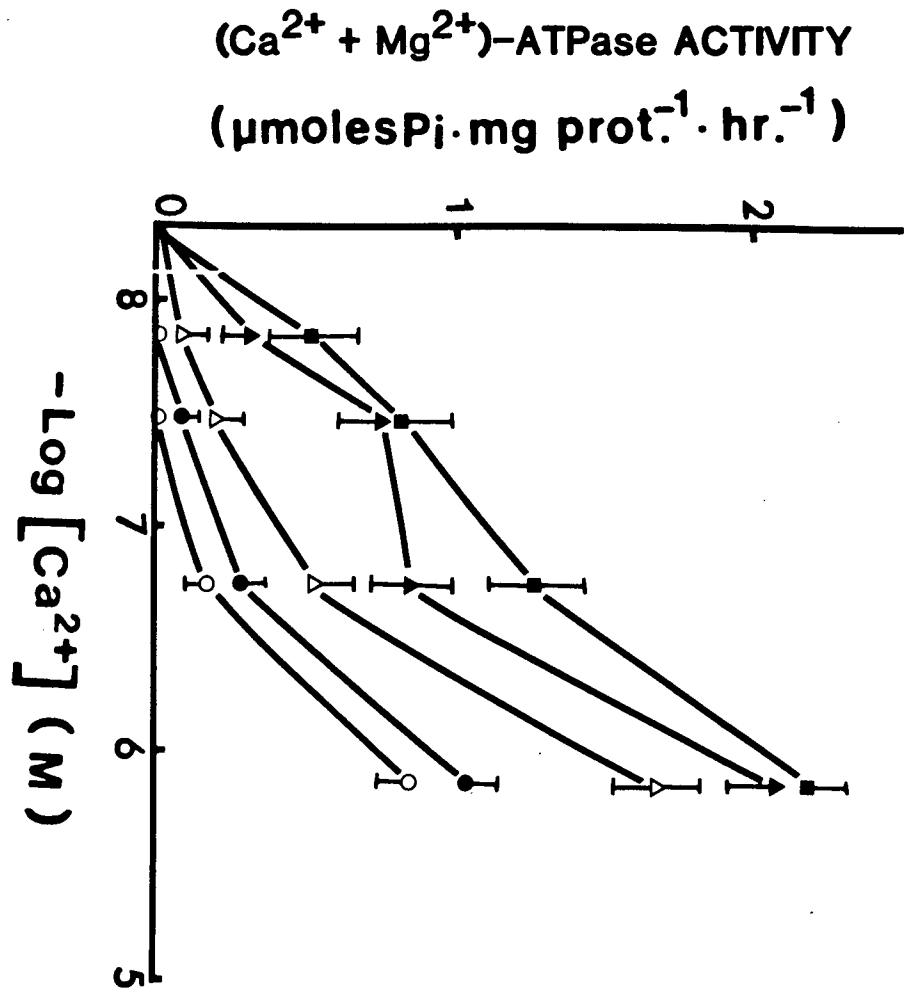
increase in activity was not due to the additive effect of Mg^{2+}-ATPase because the contribution of Mg^{2+}-ATPase had been subtracted from the total ATPase activity (Table VII). Therefore it appears that the effect of Mg^{2+} is to increase the sensitivity of the enzyme for calcium.

It was of interest to determine the effect of Mg^{2+} and calmodulin on the calcium activation curve of EDTA-treated membranes. Figure 20 indicates that calmodulin stimulation of EDTA-treated membranes was observed irrespective of the presence or absence of Mg^{2+}. This is in agreement with the previous conclusion that Mg^{2+} is required only for the expression of the Ca^{2+}-ATPase activity. Calmodulin and Mg^{2+} seem to produce a similar effect on the affinity of the enzyme for calcium.

In order to obtain information on the mechanism by which calmodulin activates the Ca^{2+}-ATPase, it is important to know if calmodulin interacts directly with the ATPase molecule. To resolve this problem, an ^{125}I-labeled calmodulin gel overlay technique was employed. This procedure indicates the ability of calmodulin to bind to denatured target proteins present on the gel. The autoradiogram shows the binding of ^{125}I-labeled calmodulin to a number of membrane proteins (Figure 21). In contrast to other calmodulin binding proteins that appeared on the autoradiogram, calmodulin binding to a 133,000 and a 230,000 dalton protein were Ca^{2+}-dependent as no binding occurred in the presence of EGTA.

Table VII

Mg^{2+}-ATPase Activity in EDTA Treated Plasma Membrane
Preparations from Pancreatic Acinar Cells.

Mg^{2+} Conc. µM	Mg^{2+}-ATPase Activity (µmoles Pi/mg prot/hr)
0.5	0.16 ± 0.07
1.0	0.23 ± 0.05
2.0	0.52 ± 0.12
5.0	2.05 ± 0.35
10.0	3.37 ± 0.66

Membrane preparations were incubated with 1 mM Tris EDTA, pH 8.0 for 30
min at 37°C. The membranes were washed twice and resuspended in
Tris-HCl buffer pH 7.4. Mg^{2+}-ATPase activity refers to the activity in
the absence of added Ca^{2+} and in the presence of various concentrations
of Mg^{2+}.

Figure 20 The effect of calmodulin and Mg^{2+} on the calcium
activation curve of EDTA treated plasma membranes of
acinar cells. Membranes were incubated with 1 mM Tris
EDTA pH 8.0 for 30 min at 37°C, washed free of EDTA and
assayed for ATPase activity. The calcium activation
curve in the absence of Mg (o), in the presence of 0.5 μM
Mg (●), 280 nM Calmodulin (Δ) and Mg^{2+} plus calmodulin
(▲) is shown. Data represent the mean ±S.E.M. of three
separate determinations.

Ca^{2+}-ATPase Activity:
µmoles Pi/mg prot/hr

−Log[Ca^{2+}] (M)
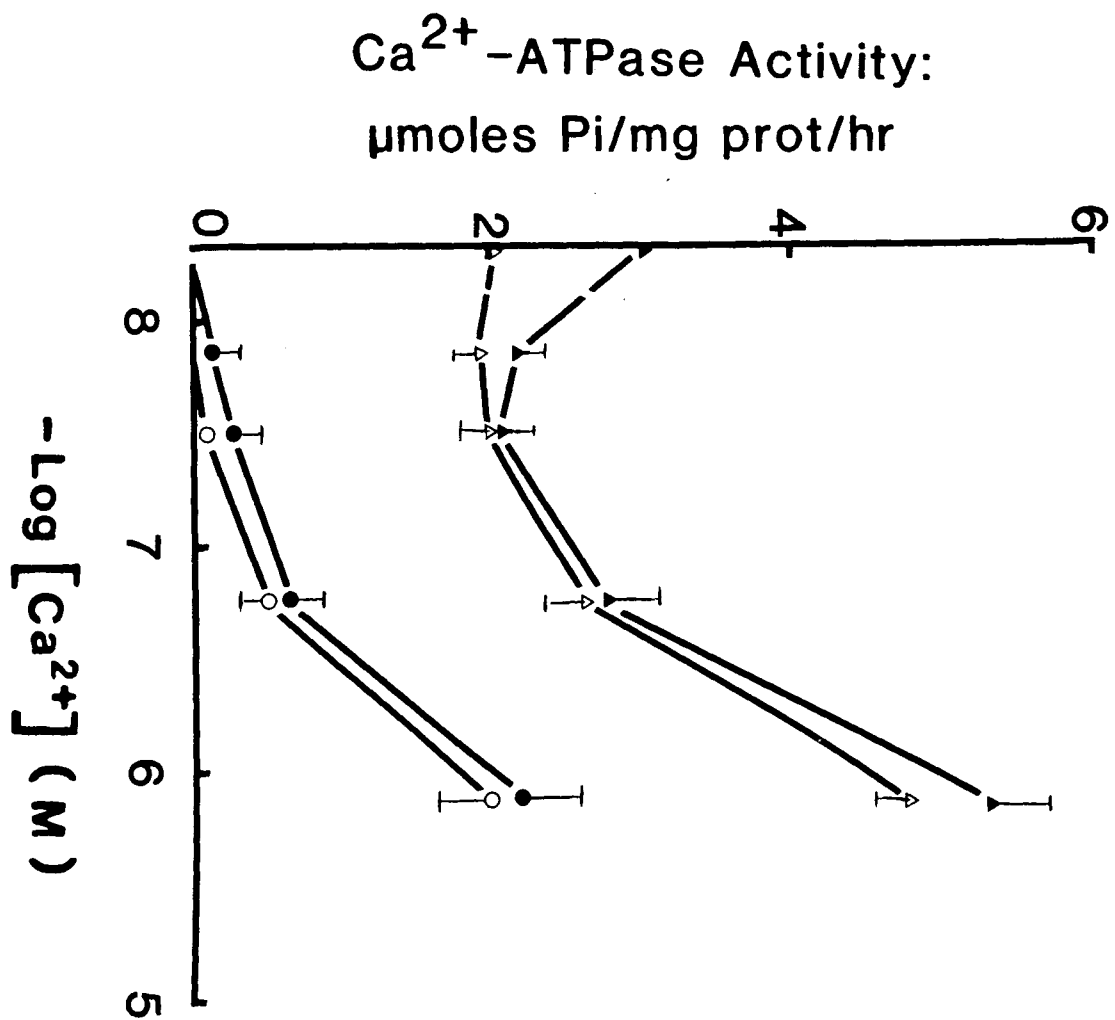
Figure 21 Calmodulin gel overlay and autoradiography of target proteins in plasma membranes of acinar cells. $[^{125}I]$-Calmodulin binding in the presence of 1 mM Ca^{2+} (Lane A) and in the absence of Ca^{2+} (5 mM EGTA; Lane B).

Mol. wt.

200 −

116 −

67 −

45 −

30 −

14 −

×10³

A   B
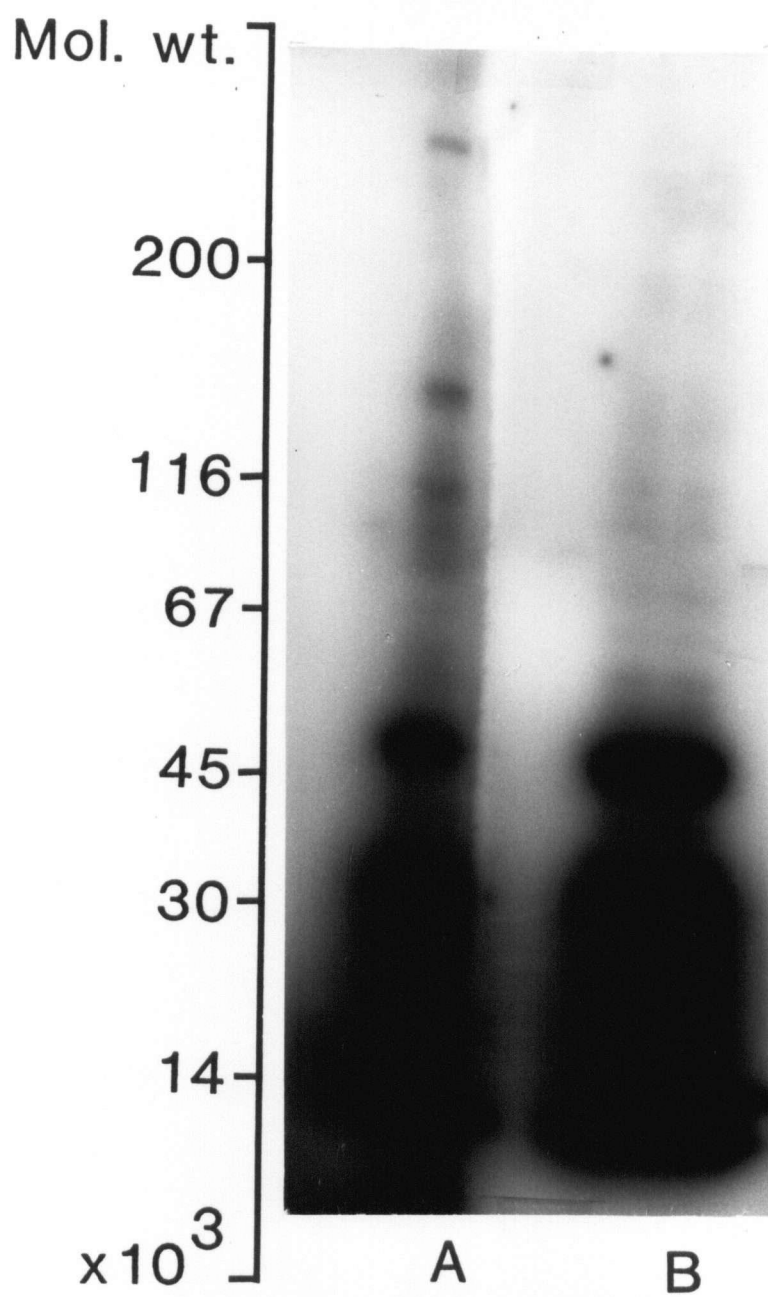
5. Regulation of Pancreatic Acinar Plasma Membrane Ca^{2+}-ATPase by Phospholipids

It has been reported that acidic phospholipids, unsaturated fatty acids and limited proteolysis mimic the effect of calmodulin on membrane-bound (Gietzen et al 1982), solubilized (Al-Jobore and Roufogalis 1981b) or purified (Niggli et al 1981) erythrocyte Ca^{2+}-ATPase. Since the pancreatic acinar plasma membrane Ca^{2+}-ATPase is activated by calmodulin, it was investigated whether phospholipids had a similar effect on this system. Figure 22 demonstrates that asolectin (a mixture of phospholipids) stimulated the Ca^{2+}-ATPase activity of acinar plasma membranes by approximately 7-fold. This activation did not require the presence of calcium. A similar activating effect was observed by Adunyah et al (1982) when purified erythrocyte Ca^{2+}-ATPase was reconstituted in asolectin. Thin-layer chromatography revealed that asolectin is composed mainly of phosphatidylcholine, phosphatidylinositol, phosphatidylserine and their lysoderivatives. When the phospholipids were tested separately, it was observed that the acidic phospholipids cardiolipin, phosphatidylserine, andphosphatidylinositol stimulated the Ca^{2+}-ATPase activity (Figure 23). As expected, phosphatidylcholine which is a neutral phospholipid failed to stimulate the ATPase activity (Figure 23). However, it was found that the pure phospholipids were not as effective as asolectin in stimulating the enzyme. It required approximately 10-fold higher pure phospholipids to produce maximum activation than that determined for solubilized erythrocyte Ca^{2+}-ATPase (Al-Jobore and Roufogalis 1981). The reason for this is not apparent but perhaps the enzyme requires a

Figure 22 Asolectin stimulation of Ca^{2+}-ATPase activity in plasma
membrane enriched fractions of acinar cells. Asolectin
stimulation in the presence (•) and absence (o) of 0.4 μM
Ca^{2+}. Results represent mean ±S.E.M. of three separate
determinations.

Ca^{2+}-ATPase Activity: μmoles Pi/mg prot/hr

Asolectin Conc. (μg/ml)

- 120 -
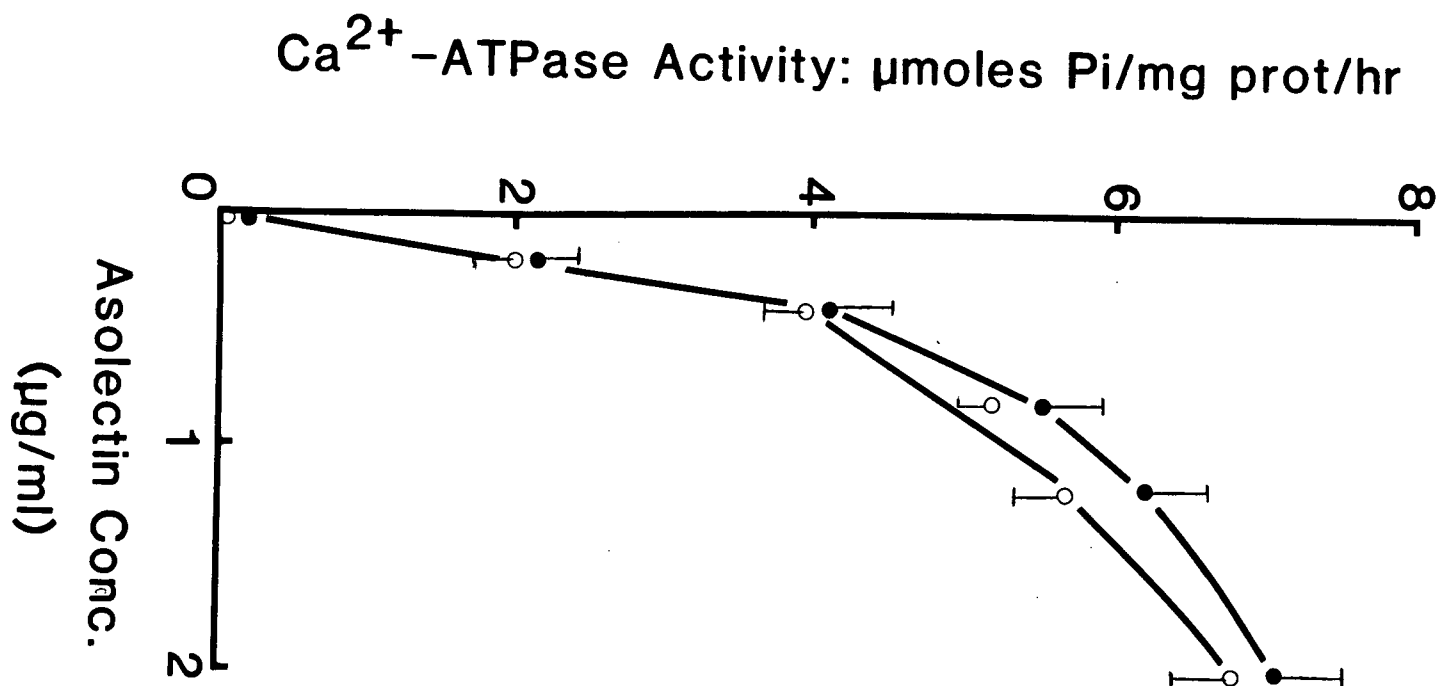
Figure 23 The effect of exogenous phospholipids on Ca^{2+}-ATPase
activity in plasma membrane enriched fractions of acinar
cells. Enzyme activity was assayed in the presence of
phosphatidylcholine (O---O), phosphatidylinositol
(●——●), phosphatidylserine   (✻——✻) and cardiolipin
(▲——▲). Data represent the mean of two different
experiments each in duplicates.
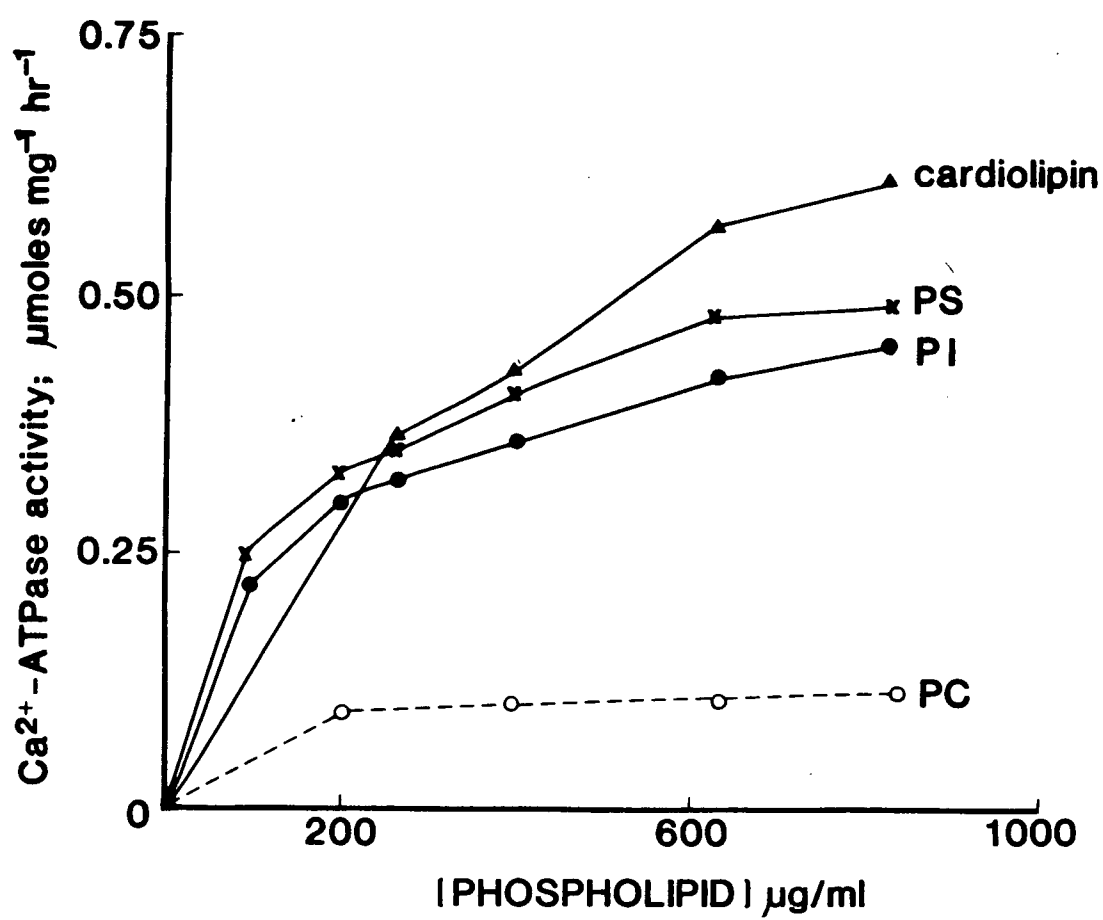
milieu of different phospholipids rather than a specific phospholipid for optimum activation.

Thus far it has been demonstrated that the acinar plasma membrane Ca^{2+}-ATPase has a number of properties characteristic of the Ca^{2+}-transport ATPase in other systems. A common feature of these ATPases is the formation of a Ca^{2+}-dependent labile phosphorylated intermediate. An autoradiogram of experiments designed to identify the hydroxylamine sensitive acyl-phosphate intermediate is shown in Figure 24. In the absence of Ca^{2+} (in the presence of 0.4 mM EGTA) no phosphorylated intermediate was formed (Figure 24 lane A). Addition of 80 μM Ca^{2+} resulted in the formation of a phosphorylated intermediate in the region of 115,000 daltons (Figure 24 lane B). In the presence of Mg^{2+} alone (in the presence of endogenous Ca^{2+}), or in the presence of Ca^{2+} and Mg^{2+}, two phosphorylated intermediates were formed (M_r 132,000 and 115,000 Figure 24, lane C and D). Both phosphoproteins were unstable in the presence of hydroxylamine (not shown).

It has been known for sometime that membrane phosphorylation may regulate certain cellular functions. Membrane phosphorylation can be catalysed by protein kinase enzymes associated with the membrane or by cytoplasmic protein kinases. In this experiment attempts were made to identify any membrane proteins phosphorylated by endogenous protein kinases under various experimental conditions. Standard procedures for measuring protein kinase phosphorylation were utilized, such as a high

Figure 24   SDS polyacrylamide gel electrophoresis and
            autoradiography of acyl phosphate intermediates of
            pancreatic acinar plasma membranes. ^{32}p-bound to
            phosphoproteins separated on 8% slab gel. A lane: no
            added ions (presence of EGTA); B lane; Ca^{2+} (80 µM); C
            lane: Mg^{2+} (80 µM); D lane $Ca^{2+} + Mg^{2+}$ (80 µM and 80
            µM).
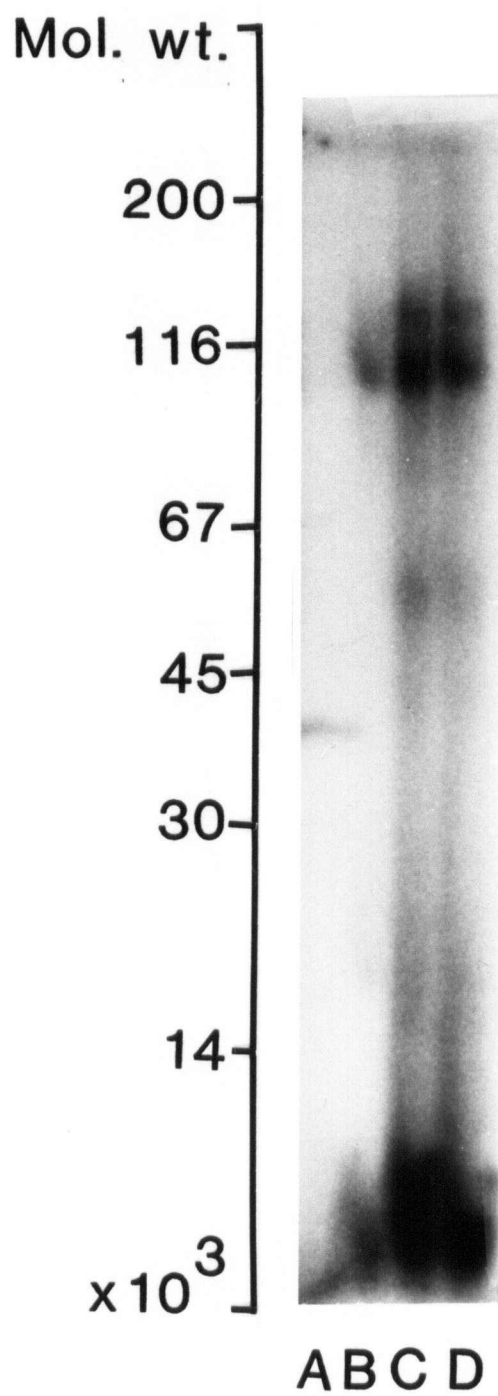
ATP concentration (0.5 mM) and a longer incubation time (5 min). As shown in Figure 25, similar phosphorylation patterns were observed both in the presence and absence of Ca^{2+}. However calmodulin in the presence of Ca^{2+} produced a greater degree of phosphorylation of a 19,000 and a 30,000 dalton protein (Figure 25 lane C).

The low molecular bands at the gel front (Figure 24 and 25) may represent phosphorylation of membrane lipids. Enyedi et al (1983) has reported the phosphorylation of polyphosphoinosites in lymphocyte, platelet and red cell plasma membranes by cyclic AMP-dependent protein kinase. This suggests that the phosphorylation of membrane lipids may play a role in the regulation of cellular functions.

6. Determination of the Presence of a Ca^{2+}-Transport Process in Acinar Plasma Membrane Vesicle Preparations

Many Ca^{2+}-transport processes are mediated by a membrane bound high affinity Ca^{2+}-ATPase activity (Penniston 1982). Therefore it was investigated whether an ATP-dependent Ca^{2+}-transport process existed in plasma membranes obtained from pancreatic acinar cells. For these studies, plasma membranes were resuspended in 0.3 M sucrose to favour vesicle formation.

In view of the uncertainty of the 'sidedness' of the $(Mg^{2+} + Ca^{2+})$-ATPase at this stage of the studies and the controversy existing in the literature, it was decided that both ATP-dependent Ca^{2+} efflux from pre-loaded vesicles and ATP-dependent Ca^{2+}-influx should be measured.

Figure 25   SDS polyacrylamide gel electrophoresis and
            autoradiography of phosphorylated pancreatic acinar
            plasma membranes. ^{32}P-phosphorylated proteins separated
            on a gradient (5-20%) gel. A lane: no added ions (2.2
            mM EGTA); B lane: Ca^{2+} (90 μM); C lane: Ca^{2+} +
            Calmodulin (90 μM and 100 nM); D lane; calmodulin + EGTA
            (100 nM and 2.2 mM); E lane: no added ions.

Mol. wt.

200 -

116 -

67 -

45 -

30 -

14 -

$\times 10^3$

A B C D E
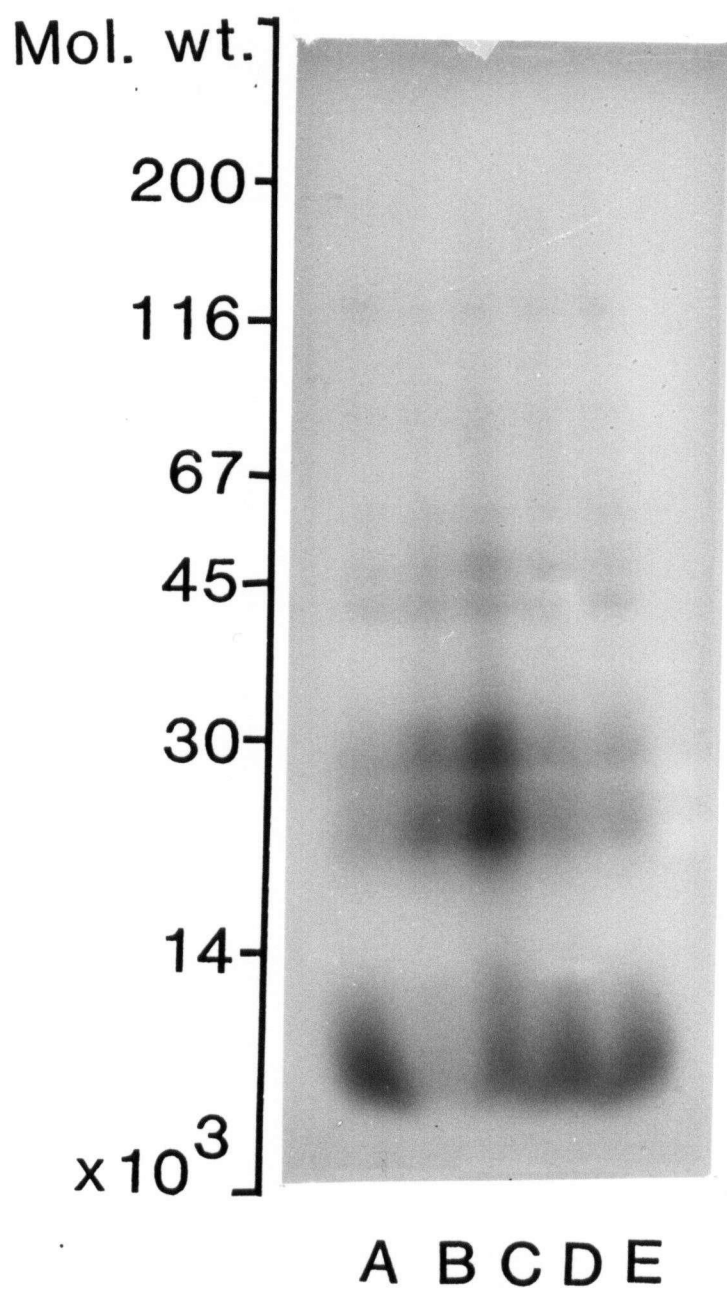
In preliminary experiments, it was observed that the amount of $^{45}Ca^{2+}$ associated with the vesicles was consistently lower when vesicles were loaded in the presence of ATP than when ATP was absent. This suggested that perhaps there was an ATP-dependent Ca^{2+}-extrusion process involved. A series of experiments were then performed in which ^{45}Ca efflux from preloaded vesicles was determined. Figure 26 illustrates the presence of an ATP-dependent Ca^{2+} extrusion system in the membrane vesicles, followed by a slow but steady uptake. The obvious concern at this point was whether the initial Ca^{2+} content of the vesicles in the presence or absence of ATP was similar, as would be expected if Ca^{2+} extrusion was the only existing process. To test this, oxalate was included in the preloading medium in order to prevent Ca^{2+}-efflux. As shown in Figure 27, Ca^{2+}-efflux was greatly reduced under these conditions. However, the ^{45}Ca content of the vesicles preloaded in the presence of ATP was much higher, suggesting the existence of an ATP-dependent accumulation process. ATP dependent Ca^{2+}-uptake was therefore measured. In these studies vesicles were formed in the absence of ATP and ^{45}Ca and then these agents were added to the incubation media. The Ca^{2+}-uptake observed was rapid, reaching a maximum in 2 min (Figure 28). The accumulated Ca^{2+} could be released by the calcium ionophore A23187 (5 μM). It was observed that transferring the vesicles to an EGTA containing buffer prior to filtration increased the uptake rates. The EGTA washing step to remove bound Ca^{2+} was incorporated in subsequent experiments.

Figure 26 ^{45}Ca fluxes in preloaded plasma membrane vesicles prepared from pancreatic acinar cells. Calcium loaded vesicles in the presence (●━━●) and absence (○---○) of 5 mM ATP at 0°C were resuspended in non-radioactive medium and the transport assay performed at 37°C. The result is the mean ± S.E.M. of three determinations.
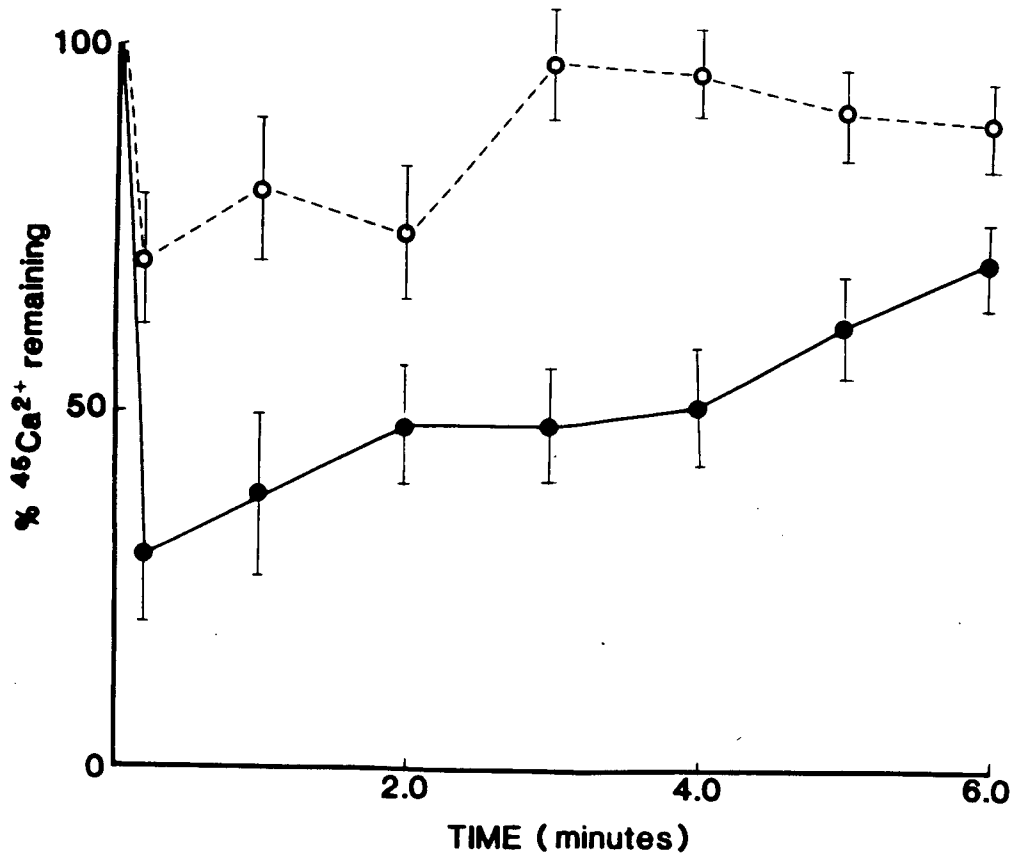
Figure 27 ^{45}Ca content of preloaded plasma membrane vesicles prepared from pancreatic acinar cells. Oxalate facilitated calcium loaded vesicles in the presence (●) and absence (o) of 5 mM ATP at 0°C were resuspended in non-radioactive medium and transport assay performed at 37°C. Results represent the mean ± S.E.M. of three determinations.

2+,9
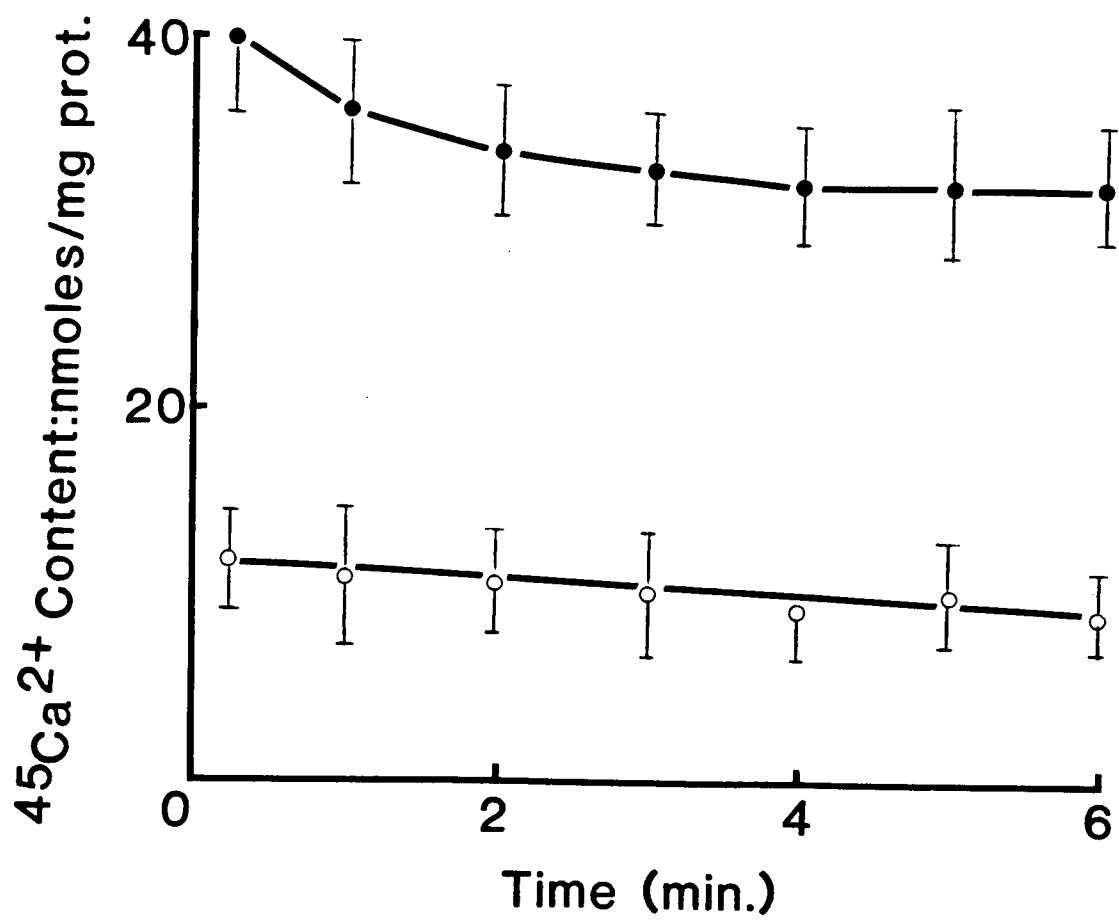
Figure 28   Time-dependent Ca^{2+}-uptake in pancreatic acinar plasma membrane vesicles. Ca^{2+}-uptake in a medium containing 75 μM Ca^{2+} in the presence ($\bullet$——$\bullet$) and absence (o....o) of 5 mM ATP.

The vesicles were pretreated with 2 mM EGTA prior to the transport assay. Results represent the mean ± S.E.M. of four different experiments.
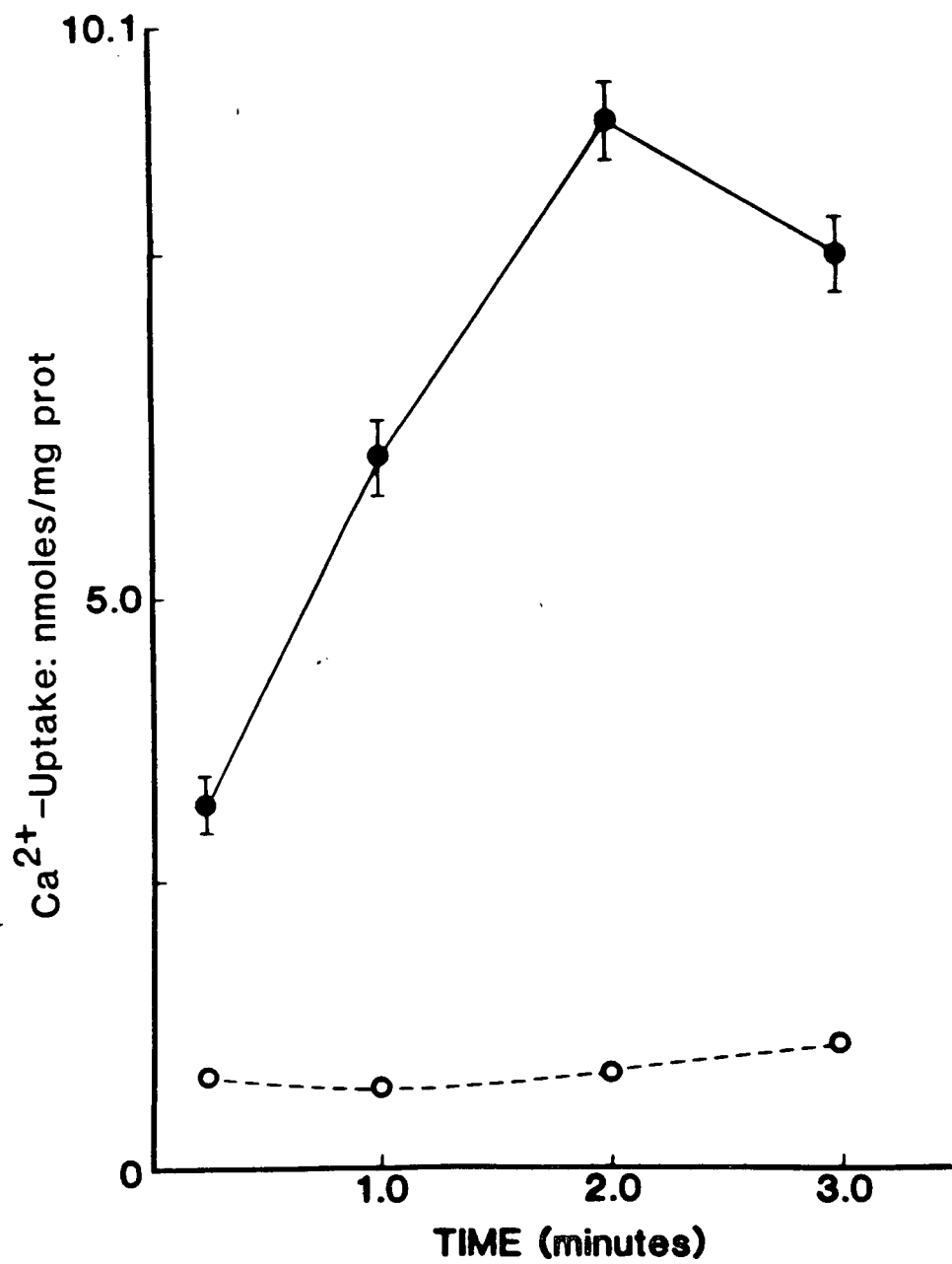
Figure 29 represents the effect of Mg^{2+} on Ca^{2+}-uptake activity at varying free Ca^{2+} concentrations. Addition of Mg^{2+} produced a marked increase in Ca^{2+}-uptake activity indicating that Mg^{2+} is essential for the Ca^{2+}-uptake process.

7. Determination of the Orientation of the Catalytic Site of the Ca^{2+}-ATPase Activity in the Plasma Membranes of Acinar Cells

In order to relate the $(Mg^{2+} + Ca^{2+})$-ATPase activity observed in plasma membrane preparations to a possible functional role in the acinar cell it was important to determine the orientation of the catalytic site of this enzyme. With the availabilty of dispersed pancreatic acinar cells it was possible to measure the ATP hydrolytic activity of the cells suspended in the incubation medium. If the active site of the Ca^{2+}-ATPase faces the cytoplasm as in a majority of Ca^{2+}-transport ATPases, no ATP hydrolytic activity would be observed. On the contrary, a high ATP hydrolytic activity was observed in the presence of either Ca^{2+} or Mg^{2+} (Figure 30). The ATPase activity remained largely unchanged after disruption of the cells by freezing and thawing indicating that greater than 90% of the activity is externally-oriented (Table VIII). Table VIII indicates that the ATPase activity observed in intact cells was not due mainly to broken or damaged cells since the cytoplasmic enzyme marker, lactate dehydrogenase was hardly detectable (0.005% of total) in these preparations.

An investigation of the 'sidedness' of the $(Mg^{2+} + Ca^{2+})$-ATPase was also carried out in the vesicle preparation used to measure

Figure 29 ATP-dependent Ca^{2+}-uptake as a function of
Ca^{2+}-concentration in pancreatic acinar membrane
vesicles.
Ca^{2+}-uptake in the presence (●——●) and absence (○---○)
of 5 mM Mg^{2+} added to the incubation medium following
vesicle sealing. The vesicles were pretreated with 2 mM
EGTA prior to the transport assay. Data represent the
mean ± S.E.M. of four different experiments.
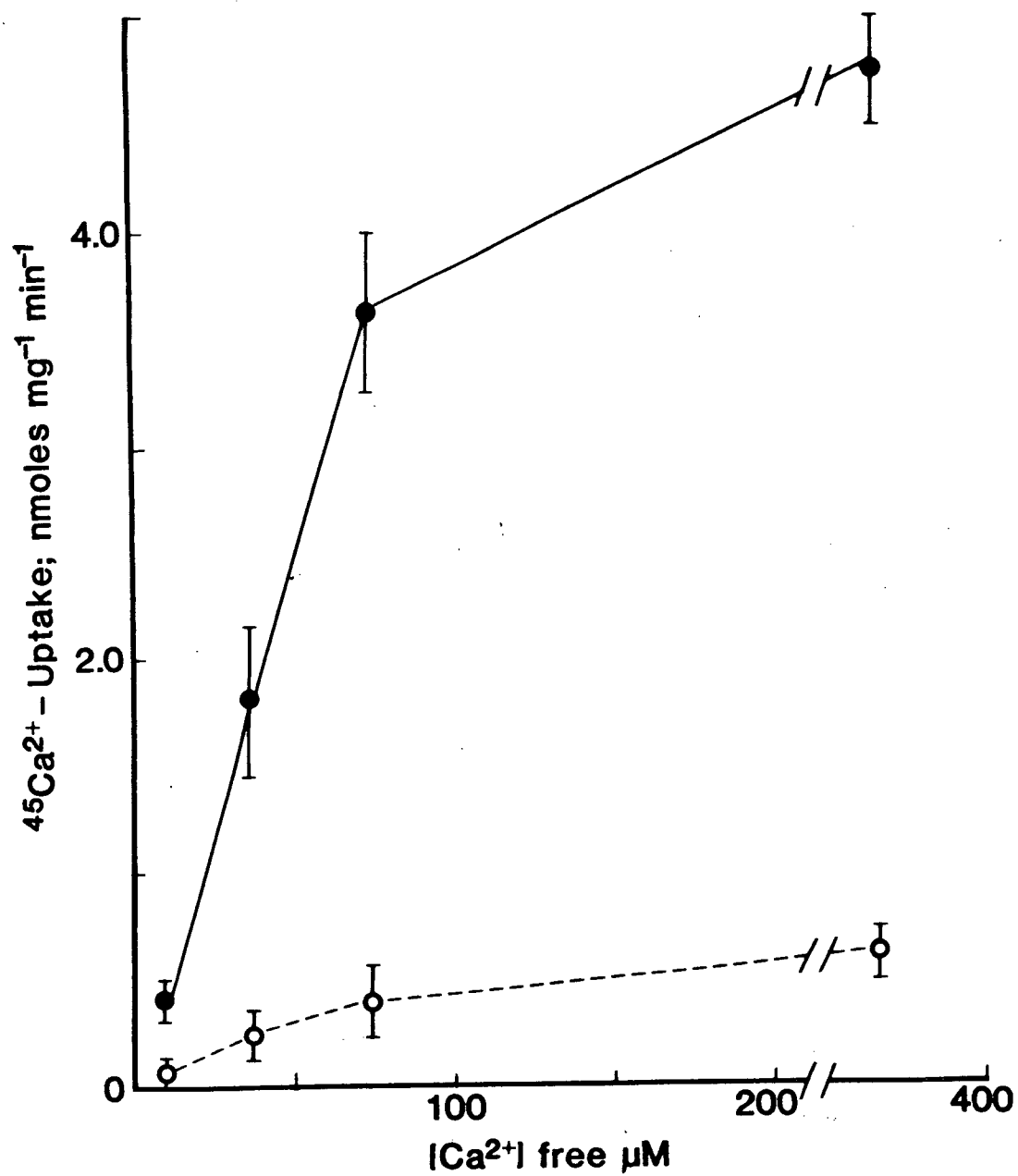
Figure 30 ATPase activity of intact pancreatic acinar cells and
homogenates. ATP hydrolytic activity in cell suspension
(10^6 cells/ml) in the presence of Ca^{2+} (●——●) and Mg^{2+}
(▲——▲) or cell homogenates in the presence of Ca^{2+}
(O---O) and Mg^{2+} (△---△). Data represent the mean ±
S.E.M. of four different determinations.
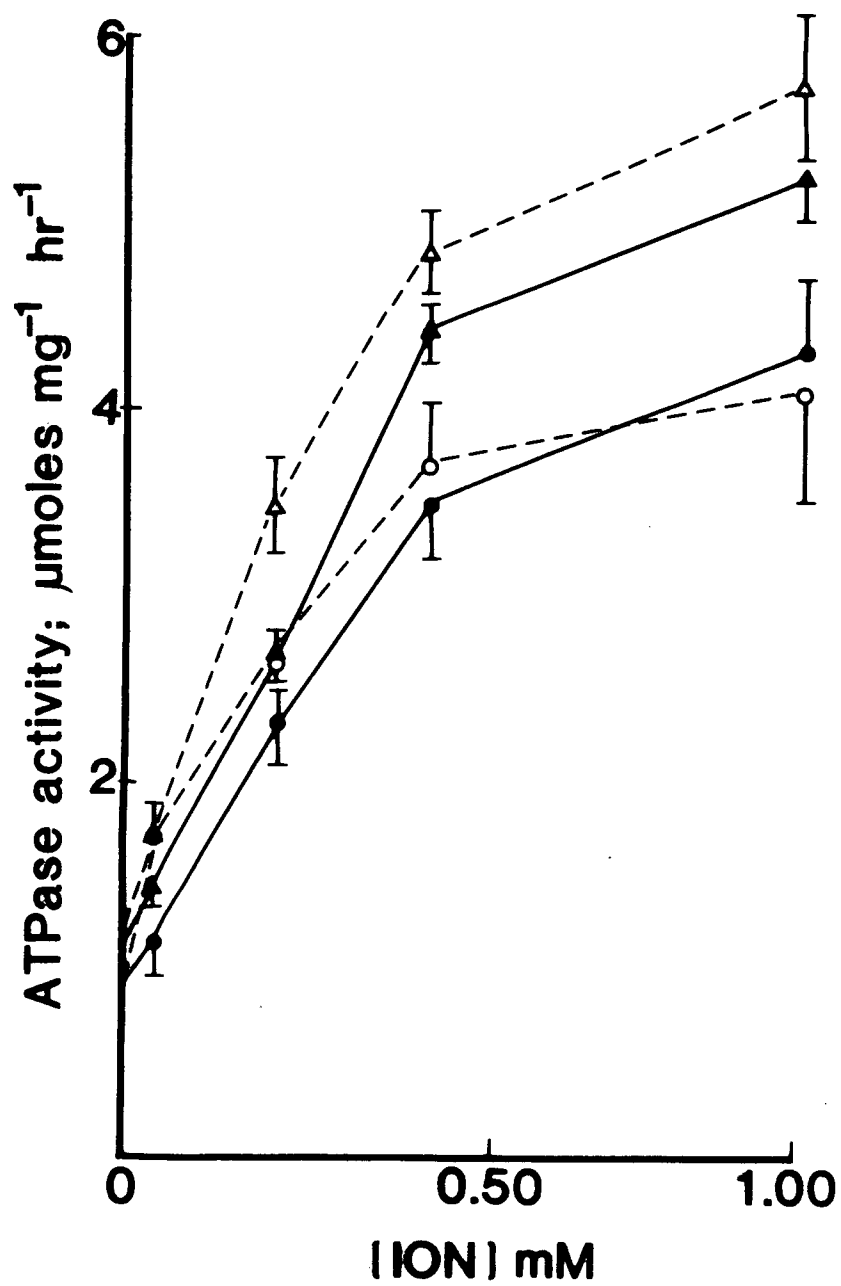
Table VIII

ATPase and Lactate Dehydrogenase Activities of Intact Pancreatic
Acinar Cells and Homogenates.

	Specific Activity (μmoles/mg prot/hr)	
	Intact Cells	Cell Homogenates
Ca^{2+}-ATPase	4.26 ± 0.6	4.16 ± 0.8
Mg^{2+}-ATPase	5.22 ± 0.3	5.78 ± 0.5
Lactate dehydrogenase	0.01 ± 0.0	1.81 ± 0.5

Intact acinar cells were resuspended in phosphate free Krebs-Ringer-
bicarbonate solution containing 2 mM ATP, 5 mM NaN$_3$ and 1 mM Ca^{2+} or
Mg^{2+} at a concentration of 10^6 cells/ml for 15 min at 37°C. A portion
of the cell suspension was similarly treated except that it was
subjected to freezing and thawing before enzyme assay (cell
homogenates). In both cases aliquots of the incubation media were
centrifuged at 10,000 rpm for 45 sec and the supernatant assayed for
inorganic phosphate or lactate dehydrogenase activity. Specific
activity is expressed as μmoles per mg cell protein. Results represent
mean ± S.E.M. of four different experiments.

ATP-dependent Ca^{2+}-transport (see the previous section). A similar orientation as that noted in the intact cells was observed; the vesicles exhibited an accessible $(Mg^{2+} + Ca^{2+})$-ATPase activity comparable with that of broken membranes (Table IX). This indicates that the majority of these vesicles were right-side out (> 95%). Consistent with a fully accessible catalytic site, neither deoxycholate (0.04 - 0.25 mM) nor freezing and thawing could unmask latent activity (Table IX).

Table IX

The Orientation of Ca^{2+}-ATPase Activity in Plasma
Membrane Vesicle Preparations.

Treatment	% Control
Control	100
DOC 40 μM	101.3
80 μM	103.8
120 μM	98.7
160 μM	98.5
200 μM	91.0
240 μM	90.7
Freeze thaw 1x	101.2
Freeze thaw 2x	97.5

Plasma membrane vesicle preparations were incubated in 20 mM Tris buffer
pH 7.4 containing 0.3 M sucrose, 2 mM NaN_3, 0.5 mM ATP, 21 μM free Ca^{2+}
and in the presence of various concentrations of deoxycholate (DOC).
Data represent the mean of two different experiments, each done in
duplicate.

DISCUSSION

A Ca^{2+}-ATPase activity has been characterized in plasma membrane enriched preparations from pancreatic acinar cells. Its subcellular distribution parallels that of plasma membrane marker enzymes, suggesting that the Ca^{2+}-ATPase activity is endogenous to the plasma membrane.

Various workers (Forget and Heisler 1976; Milutinovic et al 1977; Lambert and Christophe 1978; Le Bel et al 1980; Martin and Senior 1980) have described a Ca^{2+}-ATPase activity in plasma membranes prepared from pancreatic tissue homogenates. Since the pancreas is a heterocellular organ, the Ca^{2+}-ATPase activity observed could not be directly related to acinar cell function. The present study employed a homogeneous population of acinar cells. Radioimmunoassay showed a 35 - 75% decrease in insulin levels in the final preparation. The residual insulin detected may be insulin dislodged from binding sites on acinar cells (Korc et al 1978) rather than contamination by β-cells. Cytochemical analysis revealed one cell type with features characteristics of acinar cells. The staining technique used was general enough to detect any other cell type present.

In contrast to previous studies (Forget and Heisler 1976; Milutinovic et al 1977; Lambert and Christophe 1978; Le Bel et al 1980; Martin and Senior 1980) which reported calcium sensitivity of the ATPase in the millimolar range, a high affinity Ca^{2+}-ATPase with a K_d for Ca^{2+} of 1.7 μM was observed in these plasma membrane preparations.

Since the free concentration of calcium in the acinar cell cytosol is typically between 0.1 and 10 µM, this enzyme may play a role in the regulation of intracellular free calcium concentrations. The Ca-EGTA buffer system and the low ATP concentrations (0.5 mM) used are necessary to reveal the high affinity component of the Ca^{2+}-ATPase (Lotersztajn et al 1981). These conditions were not employed by previous workers and could explain why the high affinity component was not observed in their studies.

By the use of EDTA-treated membranes, an apparent requirement of this Ca^{2+}-ATPase activity for Mg^{2+} was demonstrated. Presumably, endogenous or tightly bound Mg^{2+} is sufficient to satisfy the Mg^{2+} requirement of this Ca^{2+}-ATPase. The plasma membrane Ca^{2+}-ATPase of cells from corpus luteum (Verma and Penniston 1981), liver (Lotersztajn et al 1981) pancreatic islets (Kotagal et al 1982) and embryonic bone cells in culture (Shen et al 1983) have been shown to have similar Mg^{2+} requirements. Increasing the Mg^{2+} concentration increases the ATPase activity, eventually reaching ATP hydrolytic rates similar to that observed in the presence of Ca^{2+} alone. Evidence obtained indicates that the activity measured in the presence of Ca^{2+} or Mg^{2+} is one enzyme with a catalytic site that has different affinities for the cations: First, saturating concentrations of Ca^{2+} and Mg^{2+} produce the same maximum activity. Second, both Ca^{2+}-ATPase and Mg^{2+}-ATPase activities have similar pH optima. Third, there is a striking similarity in their substrate requirement profile. However, differences observed in transition temperatures for the Mg^{2+} and Ca^{2+} activity and the possible

presence of different phosphoenzyme intermediates cannot be explained on the basis of a single enzyme.

The acinar plasma membrane Ca^{2+}-ATPase activity was stimulated by calmodulin; this stimulation was blocked by micromolar concentrations of trifluoperazine and chlorpromazine. The concentrations of trifluoperazine (30 μM) and chlorpromazine (55 μM) that produced half maximal inhibition of the calmodulin stimulated activity are similar to those reported for other systems (Roufogalis 1982). Although the concentrations of calmodulin used were high (K_d = 750 nM) compared to those required for activation of phosphodiesterase and erythrocyte Ca^{2+}-ATPase (Scharff 1981), much higher concentrations of calmodulin are required for the activation of the Ca^{2+}-ATPase of islet cell plasma membrane (Kotagal et al 1982) and canine ventricle sarcoplasmic reticulum (Kirchberger and Antonetz 1982). The calmodulin (42 ng/mg protein) that remained bound to the membranes during isolation could explain this decreased responsiveness. The saturating concentration of calmodulin used (1.7 μM) is only a fraction of the total amount of calmodulin in the cytoplasm of acinar cells (estimated to be 5 μM; Vandermeers et al 1977). Calmodulin stimulation of the Ca^{2+}-ATPase observed in the absence of added calcium (Table VI) could be due to the presence of endogenous calcium; under the condition of the assay, this is estimated to be 9nM (7 μM total). In the presence of 1 mM EGTA, only 20% of the calmodulin activation was lost, suggesting that perhaps not all endogenous Ca^{2+} was chelated. The EGTA concentrations were not further increased to avoid the direct effect of EGTA on ATPase activity

observed by others (Sarkadi et al 1979; Al-Jobore and Roufogalis 1981a; Kotagal et al 1983). The lack of calmodulin stimulation in the presence of 1 mM EDTA can be related to the fact that Mg^{2+} confers Ca^{2+} sensitivity to the ATPase activity as shown by experiments with EDTA-treated membranes (Fig 19).

Experiments performed to determine the effect of calmodulin on EDTA-treated membranes revealed complex kinetics (Fig. 20). Calmodulin stimulation was observed both in the absence of added Ca^{2+} and in the presence of Mg^{2+} alone. The degree of stimulation was found to be consistently lower upon addition of 0.015 μM Ca^{2+} in both cases. In light of the observations of Kotagal et al (1983) indicating that Ca-EGTA stimulates pancreatic islet plasma membrane Ca^{2+}-ATPase, it is possible that the Ca-EGTA buffer used may have produced direct effects on the ATPase under these conditions.

The ^{125}I-labeled calmodulin gel overlay technique revealed a Ca^{2+}-dependent binding to a 133,000 dalton protein. The molecular weight of this protein compares favourably with that of erythrocyte Ca^{2+}-ATPase, reported to be 138,000 (Carafoli et al 1982). The presence of a 133,000 dalton calmodulin binding protein, coupled to the identification of a hydroxylamine sensitive phosphoprotein of similar molecular weight observed in the presence of Ca^{2+} and Mg^{2+} in this study provides convincing evidence that the 133,000 dalton protein is the Ca^{2+}-ATPase molecule, and that like many other calmodulin dependent enzymes it is stimulated by a direct interaction with calmodulin.

Tawata and Field (1982) have reported the presence of 220,000 and

150,000 dalton calmodulin binding proteins in rat liver and bovine

thyroid plasma membranes but not in erythrocyte plasma membranes. These

calcium-dependent calmodulin binding proteins appear to be components of

the cytoskeleton since they remained in the pellet after treatment of

the membranes with Triton X-100. Interestingly, a similar calmodulin

binding protein (M_r = 230,000) was observed in pancreatic acinar

plasma membranes (Fig. 21). This protein may interact with other

components of the cytoskeleton to regulate exocytosis in the exocrine

pancreas. It must be noted, though, that several calcium-independent

calmodulin-binding proteins were present in pancreatic acinar plasma

membranes, (presence of endogenous Ca^{2+} and EGTA). The function of

these calmodulin binding proteins is not known. It has been shown that

during the purification of erythrocyte (Carafoli et al 1982) and heart

sarcolemma (Caroni et al 1982) Ca^{2+}-ATPase, a large proportion of the

proteins that bind to the calmodulin affinity column can be eluted with

a Ca^{2+} wash.

In intact acinar cells it was shown that phenothiazines inhibit

calcium mediated amylase release, possibly by acting on a

calmodulin-regulated step in the stimulus-secretion coupling process.

This corroborates the observations of Heisler et al (1981) who have

suggested that calmodulin may have a functional role in exocrine

pancreatic enzyme secretion. At higher concentrations (10^{-4} M or

greater), phenothiazines may have other effects on the function of

intact cells that may account for the inhibitory effects on pancreatic

amylase secretion as indicated by Williams et al (1977) and Singh

(1980). Phenothiazines are hydrophobic molecules which partition
non-selectively into membranes and other hydrophobic sites, with
possible consequences other than antagonism of Ca^{2+}-dependent enzyme
systems. Phenothiazines can inhibit $(Na^{+}+K^{+})$-ATPase which may affect
ion gradients or tubulin, which in turn could affect the distribution of
membrane-bound receptors and secretion processes (Roufogalis 1982). In
addition, the phenothiazines have α-adrenergic and muscarinic receptor
blocking activities (Roufogalis 1982) which may complicate the
biological effects of these agents. Therefore, data obtained on
isolated tissue preparations should be interpreted with caution.
Nevertheless, under controlled conditions, the inhibitory effects of
phenothiazines have been used to invoke the role of calmodulin in the
function of a number of cells including pancreatic islets (Gagliardino
et al 1980), anterior pituitary cells (Meritt et al 1982) and mast cells
(Douglas and Nemeth 1982). Clearly, more specific anticalmodulin agents
are needed for studies involving isolated tissue preparations. Some of
the more specific anticalmodulin agents cited in the literature are
calmidazolium (Belle 1981), W7 (Hidaka and Tanaka 1982) and Compound
48/80 (Gietzen et al 1983).

In the pancreas, calmodulin has been shown to stimulate not only the
plasma membrane Ca^{2+}-ATPase activity (see above) but also cyclic
nucleotide phosphodiesterase (Vandermeers et al 1983). In view of the
fact that the role of cyclic nucleotides in calcium-mediated enzyme
release from the exocrine pancreas is still not conclusive, the
mechanism by which calmodulin activation of phosphodiesterase regulates

pancreatic enzyme secretion is uncertain.

The role of protein phosphorylation in the regulation of pancreatic function has been examined recently. Burnham and Williams (1982) have shown that carbachol and CCK8 at concentrations that produce amylase secretion increased the phosphorylation of a M_r = 32,000 particulate protein and M_r = 16,000 and 23,000 soluble proteins. These agents also altered the phosphorylation of M_r = 21,000 and 20,500 soluble proteins. Since insulin which does not produce direct stimulation of amylase release did not alter the phosphorylation of the 21,000 and 20,500 dalton soluble proteins, these authors concluded that the two proteins are specifically involved in the process of exocytosis (Burnham and Williams 1982). More recently, a phospholipid-sensitive Ca^{2+}-dependent protein kinase and its endogenous substrate proteins (M_r = 38,000, 30,000, 22,000 and 15,000) have been demonstrated in pancreatic acinar cell extracts (Wrenn 1983). Calmodulin was partially effective as a cofactor for phosphorylation of the 38,000 substrate protein (Wrenn 1983). These authors examined phosphorylation reactions in either total particulate (Burnham and Williams 1982) or soluble fractions (Burnham and Williams 1982; Wrenn 1983) with no reference to the subcellular origin of the substrate.

In these present studies when pancreatic acinar plasma membranes are incubated in the presence of Ca^{2+}, Mg^{2+} and 0.5 mM ATP for 5 min at 30°C, covalent phosphoprotein formation was observed in a number of proteins (Fig. 25). The degree of phosphorylaton of the 19,000 and 30,000 dalton proteins was enhanced by calmodulin. Of particular

interest is the similarity of this 30,000 dalton protein phosphorylation to the reported phosphorylation of a 32,000 dalton particulate protein upon stimulation of acinar cells with carbachol and CCK8 (Burnham and Williams 1982). This latter result provides a possible link between phosphorylation of plasma membrane proteins and the response of acinar cells to secretagogues.

The effect of calmodulin on pancreatic enzyme secretion can thus be envisaged as three-fold: Calmodulin could act on a plasma membrane calcium pump to regulate intracellular calcium, regulate the secretory process by interacting with the cytoskeleton via the high molecular weight calmodulin binding protein observed, or enhance phosphorylation of target proteins in the plasma membrane.

The ATPase activity described in this present study has certain characteristics similar to the ATP diphosphohydrolase present in a number of tissues. The enzyme can hydrolyse nucleoside triphosphates (ATP, GTP) and diphosphate (ADP) at high rates in the presence of Ca^{2+} or Mg^{2+}. The lack of sensitivity to azide and monovalent cations (this study), as well as ouabain, ruthenium red, oligomycin (Le Bel et al 1980) and vanadate (Martin and Senior 1980) distinguishes the ATP diphosphohydrolase from other ATPases. A similar enzyme has been characterized in a number of tissues including the liver (Garnett et al 1976), kidney (Saffran and Kinne 1974), mast cells (Cooper and Stanworth 1976), skeletal (Malouf and Meissner 1979), Smooth (Kwan 1982) and cardiac muscles (Anand-Srivastara et al 1982) and mouse sarcomas, human astrocytoma, oat cell carcinoma and melanoma (Knowles, et al 1983). The

function of this enzyme system is largely unknown. Kwan (1982) has shown that the Mg^{2+} or Ca^{2+} ATPase activity of plasma membranes isolated from vascular smooth muscle was decreased in spontaneously hypertensive rats. In rat heart sarcolemma, it has been proposed that the Ca^{2+}-ATPase component may be involved in opening of calcium channels (Anand-Srivastava et al 1982).

A feature of some of the ATP diphosphohydrolases is an externally-oriented active site. Using isolated intact cells in this present study it was demonstrated that pancreatic acinar cells can hydrolyse externally added ATP in the presence of Ca^{2+} or Mg^{2+} (Fig 30). During the course of this study Hamlyn and Senior (1983) presented evidence to suggest that 90-95% of the pancreatic acinar plasma membrane Ca^{2+} or Mg^{2+}-ATPase activity is expressed as an ecto-enzyme. While this is in agreement with the present studies, the question still remains whether all the ATPase activity associated with the plasma membrane is externally oriented and native to the plasma membrane. Both biochemical and cytochemical studies have shown that Ca^{2+}-ATPase activity exists in zymogen granule membranes (Beaudoin et al 1980). The presence of lead deposits over the luminal surface of acinar cells and on the inner surface of zymogen granule membranes at the outset of fusion indicates that part of the plasma membrane Ca^{2+}-ATPase could be derived from zymogen granule membranes as a result of exocytosis (Beaudoin et al 1980). If the active site of the Ca^{2+}-ATPase is situated on the cytoplasmic side of the zymogen granules, then following fusion with the plasma membrane, the enzyme would be internally oriented. So far this

has not been demonstrated cytochemically. Therefore, until the mechanism of membrane fusion is fully understood the question of the distribution and origin of plasma membrane Ca^{2+}-ATPase will remain unresolved. Moreover, treatment of intact acinar cells with the slowly penetrating covalent reagent, diazotized sulphanilic acid (DSA) destroyed about 90% of the total ATPase activity present in pancreatic plasma membranes (Hamlyn and Senior 1983). This could suggest that there may be a pool of plasma membrane ATPase that was not accessible to the externally applied probe.

In addition to the zymogen granule Ca^{2+}-ATPase reported (Beaudoin et al 1980), Ponnappa et al (1981) have described a Ca^{2+}-ATPase activity associated with microsomal preparations obtained from acinar cells. It is possible that this activity represents contamination from plasma membranes present in this fraction. An indigenous Ca^{2+}-ATPase activity though may be present as the characteristics of the activity described by Ponnappa et al (1981) appear different from that activity associated with the plasma membrane observed in the current work: The microsomal Ca^{2+}-ATPase activity was not stimulated by calmodulin (Williams, personal communication) and the high Mg^{2+}-stimulation of the plasma membrane-related activity was not observed.

Thus in addition to the plasma membrane Ca^{2+}-ATPase system described, the acinar cell may also possess a microsomal Ca^{2+}-ATPase possibly associated with the endoplasmic reticulum or mitochondrial activity and a zymogen granule system.

The presence of ecto-ATPases have been reported in platelets (Chambers et al 1967), polymorphonuclear leucocytes (De Pierre and

Karnovsky 1974), mast cells (Cooper and Stanworth 1976), and rat mammary

gland cells (Carraway et al 1980). Despite their widespread occurrence,

the physiological function of the ecto-ATPases is not clear. It has

been suggested that the ecto-ATPases may provide a mechanism for the

termination of the ATP signal in processes where ATP may be acting as a

neurotransmitter (Carraway et al 1980). Recently, Gallacher (1982)

showed that external ATP can stimulate amylase release from superfused

mouse parotid glands in the presence of atropine, phentolamine and

propranolol, an effect which can be blocked by quinidine. Although this

is an interesting observation, further work is required to establish the

role of ATP as a secretagogue of amylase secretion.

It is now known that calcium fluxes during secretagogue-stimulated

enzyme secretion are biphasic. There is an initial Ca^{2+}-extrusion from

the cell due to release from intracellular stores (Matthews et al 1973;

Case and Clausen 1973), followed by a reuptake phase (Kondo and Schulz

1976 a and b) into an intracellular compartment different from the

trigger pool (Stolze and Schulz 1980). Although the presence of an

ATP-dependent calcium sequestration in mitochondria and rough

endoplasmic reticulum has been recognized (Ponnappa et al 1981; Wakasugi

et al 1982) little is known about Ca^{2+}-transport processes at the level

of the plasma membrane. In view of the bidirectional Ca^{2+} fluxes

reported in intact cells, both ATP-dependent Ca^{2+} efflux from pre-loaded

vesicles and ATP-dependent Ca^{2+}-influx were measured in these present

studies. The ^{45}Ca efflux observed in preloaded vesicles represented the

release of Ca^{2+} sequestered since La^{3+} (1.2 mM) present in the washing

medium would displace bound calcium. Moreover, Ca^{2+} efflux from preloaded vesicles was considerably reduced when vesicles were preloaded in the presence of oxalate. It was recognized, though that there was a considerable degree of calcium binding which had the tendency to obscure ATP-dependent Ca^{2+} influx. Ca^{2+}-uptake though, was consistently observed when the vesicles were transferred to an EGTA containing buffer before Millipore filtration. This ATP-dependent Ca^{2+}-uptake was abolished in the presence of calcium ionophore indicating that calcium was sequestered into vesicles and not just bound to the surface of the vesicles. Kribben et al (1983) have also reported an ATP-dependent Ca^{2+}-uptake in pancreatic plasma membrane preparations. As in the observations of Kribben et al (1983), Ca^{2+}-uptake was found to be Mg^{2+}-dependent although some level of uptake was observed in the absence of added Mg^{2+} (Fig. 29). However, the calcium concentrations used in these studies were much higher than those used by Kribben et al (1983). Ponnappa et al (1981) reported the presence of Ca^{2+}-transport processes in both microsomal and plasma membrane preparations. It thus appears that, similar to contractile systems, the regulation of cytosolic Ca^{2+} in the acinar cell may be a function of both a 'microsomal' (possibly endoplasmic reticulum) Ca^{2+}-uptake system and a plasma-membrane ATP-dependent Ca^{2+} extrusion system.

The great majority of the vesicles used in the Ca^{2+}-flux studies sealed right-side out as 95% or more of the ATPase activity appeared accessible from the outside, similar to the orientation observed in the intact cells. Based on the presence of a Ca^{2+} uptake system in the

vesicle preparations, it is tempting to speculate that such a system may also exist in intact cells. A number of problems with this assumption are worth considering. Firstly, Ca^{2+} in this situation would be moving down an electrochemical gradient so an ATP-dependent uptake would not be normally required. Secondly, the acinar membrane appears to be adequately permeable to Ca^{2+}. Therefore an active process does not appear to be necessary. Thirdly, the source of the extracellular ATP is uncertain, though there is evidence that ATP may be translocated from the cytosol to the cell exterior (Trans 1974). It is known that zymogen granules contain ATP (Grinstein, personal communication) and calmodulin (Heisler, personal communication). Therefore exocytosis of zymogen granules could release these effectors to the exterior of the acinar cell.

Calcium influx into red blood cells has been demonstrated after inhibiting the outward transport of calcium by vanadate (Verecka and Carafoli 1982). This calcium influx was shown to be carrier-mediated since it was saturable, K^+ sensitive and was inhibited by verapamil, quinidine and Co^{2+} (Verecka and Carafoli 1982). Similarly, it has been shown that provision of respiratory energy supports calcium accumulation in isolated hepatocytes (Kleineke and Stratman 1974; Dubinsky and Cockrell 1975). These observations suggest that, in resting cells, a slow cycling of Ca^{2+} across the plasma membrane occurs. This phenomenon though, has not been demonstrated in pancreatic acinar cells.

The regulatory role of calcium in cellular functions depends on the maintenance of low concentrations of cytosolic calcium. Studies by

Schulz and Heil (1979) have indicated that the Na^+/Ca^{2+} exchange system

may be responsible at least in part for calcium extrusion from acinar

cells. It is known that the secretory proteins in the pancreatic fluid

are accompanied by a constant amount of Ca^{2+} and Mg^{2+} (Beaudoin et al

1980). The Ca^{2+}-secretory protein association presumably takes place

during maturation of the secretory vesicles. Though a possible calcium

extrusion mechanism, the time course of such an event is such that it

may not be a major component of the calcium extrusion process.

The ecto-Mg^{2+} or Ca^{2+}-ATPase present in acinar preparations enriched

in plasma membranes represents the major ATP hydrolyzing activity in the

pancreas. Unlike other plasma membrane Ca^{2+}-ATPases that play an

important role in the maintenance of intracellular calcium

concentrations, this enzyme system by virtue of the orientation of its

catalytic site does not appear to share this property. The Ca^{2+}-ATPase

activity characterized, though, has many similar properties to other

Ca^{2+}-transport ATPases including stimulation by calmodulin and acidic

phospholipids, a Ca^{2+}-dependent-hydroxylamine sensitive phosphorylated

intermediate and an affinity for calcium in the micromolar range. In

addition, this activity has properties similar to other

ATP-diphospholydrolases including high Mg^{2+}-sensitivity, utilization of

ADP as substrate and the presence of its catalytic site on the outside

of the cell surface. It is not known whether the enzyme spans the

plasma membrane or if this total activity represents the presence of

both a high and low affinity system with separate functions.

The ectopic nature of the Ca^{2+} or Mg^{2+}-ATPase activity characterized

opens to question the possible mechanism(s) of regulation and function of this enzyme. For example, what would be the possible source of calmodulin and since extracellular Ca^{2+} is significantly higher than intracellular Ca^{2+}, what would be the function of a high affinity Ca^{2+}-ATPase under these conditions? One possibility is that this system functions as a signal to other events following exocytosis. Calmodulin present in zymogen granules and/or released by exocytosis may bind to Ca^{2+}-ATPase sites leading to a localized transport that may effect the next event. The Ca^{2+} levels in the secretory ducts (extracellular to acinar cells) are not constant but are altered at various stages in the secretion cycle. Therefore the activity of the Ca^{2+}-ATPase may vary depending on the environmental Ca^{2+}.

Of significant importance is the exact localization of this Ca^{2+}-ATPase: It is not known if this activity is present on the basolateral or luminal membrane of the acinar cell. The availability of this information will be of value in speculating on the interaction of the ATPase with other events in the stimulus-secretion coupling process.

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