STUDIES ON INTRACELLULAR CALCIUM POOLS IN A PANCREATIC CELL LINE

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ABSTRACT

Since the discovery that inositol 1,4,5-trisphosphate (IP_3) can release Ca^{2+} from intracellular stores, the phosphatidylinositol signal transduction system has been recognized as a major transduction pathway in most cell types. However, IP_3 is not the only intracellular Ca^{2+} mediator. In some cells, guanosine 5'-triphosphate (GTP) and arachidonic acid are known to mobilize intracellular Ca^{2+} independent of the action of IP_3 and probably play important roles in Ca^{2+} signalling. In pancreatic ductal cells the precise mechanisms of intracellular Ca^{2+} movement however are not well known. The aims of the present study are to identify and characterize intracellular Ca^{2+} pools and to investigate regulation of intracellular Ca^{2+} movement in PANC-1 cells (ductal cells of human pancreatic carcinomal origin).

Endoplasmic reticulum (ER)-enriched microsomal membrane fractions were prepared from PANC-1 cells. Ca^{2+} could be actively accumulated into the microsomes driven by an ATP-dependent Ca^{2+}-ATPase. Addition of IP_3 maximally caused a 20% release of actively accumulated Ca^{2+} which was completely blocked by heparin, an antagonist of the IP_3 receptor. Extravesicular Ca^{2+} produced an inhibition of IP_3-activated Ca^{2+} release. GTP alone stimulated a 5% Ca^{2+} release. In the presence of 3% polyethylene glycol (PEG), GTP maximally discharged 60-65% of the accumulated Ca^{2+} from the microsomal membrane fractions. The combination of GTP and IP_3 resulted in a greater release than either agent alone. The GTP effect was independent of IP_3 and not inhibited by heparin, indicating that the IP_3-activated Ca^{2+} channel is probably not involved in GTP-induced Ca^{2+} release. The release in response to GTP appeared to be mediated by an enzymatic GTP hydrolytic process since GTPγS, a nonhydrolyzable GTP analogue, had no effect on the release of Ca^{2+}. Arachidonic acid mobilized intracellular Ca^{2+} in a

concentration-dependent manner and maximally released 80% of the Ca^{2+} from the microsomes. Addition of indomethacin or nordihydroguaiaretic acid (NDGA), inhibitors of cyclooxygenase and lipoxygenase products, failed to block the action of arachidonic acid. These results indicate that arachidonic acid mediates the release of Ca^{2+} from the microsomes by a direct effect, not by its metabolites. IP_3 was ineffective in releasing any further Ca^{2+} from the microsomes following maximal Ca^{2+} release by arachidonic acid, indicating that a proportion of IP_3- and arachidonic acid-sensitive Ca^{2+} pools overlap. Other fatty acids also induced similar effects on Ca^{2+} release, suggesting that arachidonic acid-mediated Ca^{2+} release appeared to be nonspecific. Thapsigargin, an inhibitor of the intracellular Ca^{2+}-ATPase, was shown to inhibit Ca^{2+} accumulation into and induce Ca^{2+} release from PANC-1 microsomes. The thapsigargin-releasable Ca^{2+} pool included the IP_3- or arachidonic acid-sensitive pool. Studies carried out using phospholipid vesicles showed that arachidonic acid and thapsigargin did not alter membrane permeability, indicating that the arachidonic acid- and the thapsigargin-induced Ca^{2+} release was not due to either a Ca^{2+} ionophore or a membrane detergent effect. The present experiments have provided evidence for the existence of multiple non-mitochondrial Ca^{2+} pools in PANC-1 cells. These Ca^{2+} pools could be released via distinct mechanisms, in response to a variety of cellular second messengers.

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LIST OF ABBREVIATIONS

/	per
%	percent
$^{45}Ca^{2+}$	calcium-45
^{3}H	tritium-3
AA	arachidonic acid
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
BHQ	2,5-Di(tert-butyl)-1,4-benzohydroquinone
°C	degrees Centigrade
Ca^{2+}	free ionized calcium
$[Ca^{2+}]_i$	free intracellular Ca^{2+} concentration
CaM	calmodulin
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
Ci	curie
cm^2	square centimeter
cpm	counts per minute
CTP	cytidine 5'-triphosphate
DAG	diacylglycerol
ddH_2O	distilled, deionized water
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E_1,E_2	two conformational states of the enzyme
EC_{50}	a concentration of an agonist that increases an enzyme

	activity by 50%
EGTA	ethyleneglycol bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
et al.	and others
g	gram
GDP	guanosine 5'-diphosphate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-o-(3-thio) triphosphate
xg	gravitational unit
IC_{50}	a concentration of an inhibitor that reduces an enzyme activity by 50%
IP_3	*myo*-inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
ITP	inosine 5'-triphosphate
K_i	dissociation constant for inhibitor
K_m	Michealis constant
l	liter
m	milli
M	molar
min	minute
mol	mole
MW	molecular weight
n	nano
NDGA	nordihydroguaiaretic acid
PEG	polyethylene glycol
PIP_2	phosphatidylinositol 4,5-bisphosphate

PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
pS	pico siemens
sec	second
S.E.	standard error
SERCA	sarcoplasmic or endoplasmic reticulum calcium ATPase family
SR	sarcoplasmic reticulum
Tg	thapsigargin
μ	micro
U	unit
v/v	volume per unit volume
w/v	weight per unit volume

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DEDICATION

To my parents.

INTRODUCTION

I. ROLE OF CALCIUM IN STIMULUS-SECRETION COUPLING

The term *stimulus-secretion coupling*, first used by Douglas and Rubin (1961), defines the various steps that, following the interaction of a stimulus with its specific plasma membrane receptor, will eventually result in discharge of secretory products.

There is hardly any cellular function that is not influenced directly or indirectly by the universal second messenger calcium. In particular, extensive studies on the process of vesicular secretion have established the crucial role of Ca^{2+} as the triggering and controlling event. Secretion of vesicular contents by exocytosis is a common feature of electrically excitable (e.g., neurons, adrenal medullary chromaffin cells, β cells of the endocrine pancreas, cells of the adeno- and neurohypophysis) and non-excitable cells (e.g., exocrine glands, platelets, neutrophils, mast cells). It is clear that in excitable cells an increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is the triggering event inducing secretion. In non-excitable cells, secretion is presumably triggered by other second messengers, although $[Ca^{2+}]_i$ appears to act as an important modulator of the rate of secretion. Conversely, in excitable cells these second messengers may also serve a regulatory function.

In excitable cells, the rise in cellular Ca^{2+} following stimulation is largely due to Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Ozawa and Sand, 1986), however release of Ca^{2+} from intracellular stores, via the action of second messengers, predominates in non-excitable cells and may also be important in excitable cells. In addition, Ca^{2+} influx at the plasma membrane through receptor- and/or second messenger-operated Ca^{2+} channels may also prove to be important

(Meldolesi and Pozzan, 1987). In non-excitable cells the initial secretory response appears dependent on Ca^{2+} released from intracellular stores but prolonged secretion requires the presence of extracellular Ca^{2+} (Williams, 1980).

The concept that increases in intracellular Ca^{2+} stimulate secretion in non-excitable cells is supported by the following evidence: (a) Studies with fluorescent Ca^{2+} indicator dyes have revealed increases in $[Ca^{2+}]_i$ following stimulation with secretagogues in virtually every type of non-excitable cell investigated (Tsien *et al.*, 1984); (b) injection of Ca^{2+} into mast cells has been reported to induce secretion (Kanno *et al.*, 1973); (c) Ca^{2+} ionophores which bypass receptors to raise cytosolic Ca^{2+} stimulate a secretory response in mast cells (Cochrane and Douglas, 1974), neutrophils (Rubin *et al.*, 1981) and platelets (Feinman and Detwiler, 1974); and (d) depletion of cellular Ca^{2+} inhibits secretion (Williams, 1980).

Some findings, however, do not support the view that an increase in $[Ca^{2+}]_i$ induces secretion: (a) Mast cells and parotid cells have been reported to secrete at basal $[Ca^{2+}]_i$ levels following stimulation (Neher, 1988; Takemura, 1985); (b) In patch-clamped mast cells, a rise of $[Ca^{2+}]_i$ into the range produced by physiological stimulation does not elicit secretion (Penner and Neher, 1988); (c) An analysis of the dose-response curves for $[Ca^{2+}]_i$ increases and pancreatic amylase release has demonstrated that the two phenomena are clearly dissociated. With the same agonist, the threshold concentration for stimulation of enzyme release is 10-100 times lower than the threshold concentration inducing an increase in $[Ca^{2+}]_i$ (Bruzzone *et al.*, 1986). This suggests that in some non-excitable cells Ca^{2+} is neither a necessary nor sufficient stimulus for secretion.

There is convincing evidence that other second messengers, such as diacylglycerol (DAG), arachidonic acid and adenosine 3',5'-cyclic monophosphate (cAMP) serve to modulate Ca^{2+}-mediated secretion. In isolated rat pancreatic acini, Ca^{2+} ionophore and phorbol ester (or DAG analogues, which activate protein

kinase C (PKC)) cause a synergistic secretory response (Merritt and Rubin, 1985), suggesting that the complete activation of secretion requires stimulation of both Ca^{2+}-dependent and PKC-dependent pathways. Arachidonic acid and its metabolites, liberated by hydrolysis of phospholipids in secretory cells, may serve as cellular messengers to modulate secretion (Laychock and Putney, 1982). There is evidence which suggests that arachidonic acid may be involved in stimulus-secretion coupling in pancreatic islets (Turk *et al.*, 1987; Ramanadham *et al.*, 1992) and rat adenohypophysis (Knepel *et al.*, 1988). Arachidonic acid markedly stimulates the release of insulin (Turk *et al.*, 1987; Ramanadham *et al.*, 1992) and various anterior pituitary hormones (Knepel *et al.*, 1988) and causes an increase in $[Ca^{2+}]_i$ in these cells. In the neutrophil, metabolites of arachidonic acid may amplify the secretory response induced by Ca^{2+}-mobilizing agonists (Bradford and Rubin, 1985). In the parotid gland, stimulation of amylase secretion by norepinephrine requires the interaction of both Ca^{2+} and cAMP pathways to produce a full secretory response (Chaudhry and Rubin, 1990). These studies indicate that DAG, arachidonic acid and cAMP play relative roles in Ca^{2+}-mediated secretion.

II. PANCREATIC DUCTAL SECRETION

The pancreatic ductal tree is a network of branching tubules whose primary function is to conduct digestive enzymes into the duodenum. The pancreatic ductal system comprises centroacinar cells; intercalated, intralobular and interlobular (or extralobular) ducts; and a main duct. The centroacinar cells line the middle of each enzyme-secreting acinus and connect to intercalated ducts. In addition to acting as a conduit, the ducts secrete mucins (Forstner and Forstner, 1986), however, little is known about mucin biosynthesis and secretion (Forstner and

Forstner, 1986). Furthermore, the ductal cells produce a bicarbonate-rich isotonic fluid in response to stimulation with hormones (Case and Argent, 1986; Schulz, 1987). While a role for Ca^{2+} in cell secretion has been well documented, there is no direct evidence for Ca^{2+} involvement in the regulation of ductal secretion. The action of secretin is mediated via cAMP apparently not Ca^{2+} (Case and Argent, 1986), although the secretin-mediated secretion was partially inhibited by prolonged perfusion of isolated glands with a calcium free medium (Argent *et al.*, 1973). Recently, bombesin has been shown to be as effective as secretin in inducing fluid secretion from isolated ducts (Argent and Gray, 1990). In pancreatic acinar cells, bombesin also increases enzyme secretion by mobilizing cellular Ca^{2+} (Gardner and Jensen, 1986). This suggests that there may be a Ca^{2+}-stimulated pathway for ductal cells. The apical Cl^- channel, which plays an important role in ductal secretion (Argent and Gray, 1990), is regulated by changes in $[Ca^{2+}]_i$ in many types of epithelial cells (Ziyadeh and Agus, 1988). Studies on secretagogue-mediated effects of $[Ca^{2+}]_i$ have indicated that increased $[Ca^{2+}]_i$ itself may not be sufficient to induce fluid/electrolyte secretion in ductal cells (Stuenkel and Hootman, 1990).

III. REGULATION OF INTRACELLULAR CALCIUM CONCENTRATION

The effector and messenger roles of Ca^{2+} ions demand a rigorous regulation of $[Ca^{2+}]_i$. Determination of $[Ca^{2+}]_i$ by different techniques show that $[Ca^{2+}]_i$ is approximately 100-200 nM in resting cells. The inwardly directed Ca^{2+} gradient across the plasma membrane is approximately 10^4-fold. In order to maintain low $[Ca^{2+}]_i$, cells have developed an extensive network of Ca^{2+} transporting systems. In addition, cellular Ca^{2+} buffers also serve to maintain the large Ca^{2+} gradient across the plasma membrane.

1. Calcium Entry

Ca^{2+} enters cells down its electrochemical gradient. Excitable (Tsien *et al.*, 1987) and some non-excitable cells (MacVicar, 1984) express voltage-sensitive Ca^{2+} channels which allow Ca^{2+} entry into cells. In many non-excitable cells, specific Ca^{2+} channels have not yet been found. However, non-selective, Ca^{2+} permeable channels have been reported in non-excitable cells (Benham and Tsien, 1987; Lückhoff and Clapham, 1992).

Substantial evidence suggests that a depletion of the IP_3-sensitive Ca^{2+} store stimulates Ca^{2+} entry across the plasma membrane (Putney, 1990; Hoth and Penner, 1992) but the mechanisms are not clear. By combining patch-clamp and Fura-2 measurements, Hoth and Penner (1992) have identified a calcium current in mast cells that is activated by depletion of intracellular calcium stores. This appears to be the most selective calcium entry pathway yet known, as neither Ba^{2+} nor Sr^{2+} can permeate. It has been reported that at least some of the IP_3-sensitive stores are associated with the plasma membrane, possibly by cytoskeletal elements (Rossier *et al.*, 1991). In some studies, inositol 1,3,4,5-tetrakisphosphate (IP_4) is apparently involved in the regulation of Ca^{2+} entry (Irvine, 1990; Changya *et al.*, 1989; Lückhoff and Clapham, 1992), however the site and mechanism of action of IP_4 have not been sufficiently identified. Lückhoff and Clapham (1992) recently described a novel Ca^{2+}-sensitive Ca^{2+} permeable channel in endothelial cells and found IP_4 enhanced the activity of this Ca^{2+}-permeable channel.

2. Calcium Extrusion

Two Ca^{2+} extrusion pathways have been identified in cells: the plasma membrane Ca^{2+} pump and the Na^+-Ca^{2+} exchange system. Although Na^+-Ca^{2+} exchange activity has been demonstrated in plasma membrane vesicles of many non-excitable cells, the involvement of this exchanger in Ca^{2+} efflux from these

cell types is limited (Carafoli, 1987). In most cell types tested, the plasma membrane Ca^{2+} pump has a higher apparent affinity for Ca^{2+} compared to the Na^{+}-Ca^{2+} exchange system, suggesting that most Ca^{2+} efflux from cells is mediated by the plasma membrane Ca^{2+} pump (Carafoli, 1987). However, due to the lack of specific inhibitors for each of the Ca^{2+} transporting system, it is difficult to quantitate the contribution of the plasma membrane Ca^{2+} pump and Na^{+}-Ca^{2+} exchange to Ca^{2+} efflux from intact cells.

1) The Plasma Membrane Ca^{2+} Pump

Verma *et al.* (1988) have provided the most complete description of the primary structure of the plasma membrane Ca^{2+}-ATPase. cDNA coding for a plasma membrane calcium pump was isolated from a human teratoma library and sequenced. The translated sequence of the Ca^{2+}-ATPase contains 1,220 amino acids with a calculated molecular weight of 134,683 daltons. It has a calmodulin (CaM)-binding domain near the carboxyl-terminus and two domains rich in serine and threonine, one of which matches the sequence found in protein kinase substrates that are cAMP-dependent. Molecular cloning of plasma membrane Ca^{2+} pumps from rat brain (Shull and Greeb, 1988; Greeb and Shull, 1989) and from human small intestinal mucosa (Strehler *et al.*, 1990) have also been reported. These studies have demonstrated the existence of several isoforms of plasma membrane Ca^{2+} pumps, PMCA1, 2, 3 and 4. Northern blot analysis indicated that the expression of the PMCA1-4 genes is clearly organ (tissue) dependent (Greeb and Shull, 1989).

Functionally, the plasma membrane Ca^{2+}-ATPase is an E_1E_2-type enzyme, forming a phosphoenzyme that is inhibited by vanadate (Carafoli, 1986). The plasma membrane enzyme is highly dependent on CaM and ATP is the only effective substrate for the ATPase (Garrahan and Rega, 1990).

There is evidence to suggest that the plasma membrane Ca^{2+} pump is activated during hormone stimulation (Muallem *et al.*, 1988). It appears that the hormone-mediated activation of PKC is partially responsible for Ca^{2+} pump activation (Muallem *et al.*, 1988). The plasma membrane Ca^{2+} pump can also be activated by a cAMP-dependent protein kinase (Neyses *et al.*, 1985) and a cGMP-dependent protein kinase (Kai *et al.*, 1987).

2) The Na^{+}-Ca^{2+} Exchange

The Na^{+}-Ca^{2+} exchanger is an antiport system involving Ca^{2+} translocation coupled to the movement of Na^{+} ions in the opposite direction. In fact, the exchange system can operate in both directions (Philipson, 1985). There is now general agreement that Na^{+}-Ca^{2+} exchange is electrogenic with a stoichiometry of 3 Na^{+} per Ca^{2+}. The exact stoichiometry however remains controversial. In comparison with the plasma membrane Ca^{2+} pump, the Na^{+}-Ca^{2+} exchanger can be considered as a low-affinity, high-capacity Ca^{2+} pumping system. An absolute requirement for submicromolar levels of intracellular Ca^{2+} has been found for this system (DiPolo, 1979). Although ATP has been shown to significantly affect the activity of the Na^{+}-Ca^{2+} exchanger, it is not essential for its operation (DiPolo, 1989). Recently cDNA clones encoding the cardiac Na^{+}-Ca^{2+} exchange protein were identified (Nicoll *et al.*, 1990).

3. Intracellular Calcium Movement

1) The Role of Intracellular Calcium Binding Proteins

Cytosolic Ca^{2+} can be buffered by cytosolic Ca^{2+} binding molecules. Some of these Ca^{2+} binding proteins have been shown to play significant roles in cell regulation. For example, CaM, which is present in all eukaryotic cells (Heizmann

and Hunziker, 1990), regulates a number of enzymatic activities. Ca^{2+} binding proteins discovered in both ER and sarcoplasmic reticulum (SR) membranes probably contribute to the overall Ca^{2+} storage capacity of these organnelles (Milner *et al.*, 1992). They also play other important functional roles such as post-translational modification of newly synthesized proteins, a cytoskeletal function and movement of Ca^{2+} within the lumen of the SR/ER towards storage sites (Milner *et al.*, 1992). Ca^{2+} binding proteins of the ER are described in Chapter IV.

2) The Role of Intracellular Organelles

Considerable buffering of $[Ca^{2+}]_i$ is obtained by Ca^{2+} accumulation into intracellular organelles. Somlyo *et al.* (1985) reported that the total ER could contain 23-27% of the total calcium in liver cells whereas the mitochondria contain only 5% of total hepatocytic calcium. This indicates that most of the intracellular calcium is stored in the ER. Volpe and colleagues (1988) have described organelles named "calciosomes", which are different from the ER and may be the agonist-inducible Ca^{2+} store. A possible role of calciosomes in the regulation of the intracellular Ca^{2+} concentration is described in Chapter IV.

i) *Endoplasmic Reticulum*

Calcium from the ER can be released in response to the second messenger, IP_3 (Berridge, 1987). The liberated Ca^{2+} then acts as a "third messenger" by activating Ca^{2+}-dependent processes (Berridge, 1987). The ER therefore is not only the main intracellular Ca^{2+} store but also the source of IP_3-mobilizable calcium. The Ca^{2+} pool in the ER is refilled by Ca^{2+} pumps. The function of the ER is discussed in detail in Chapter IV.

ii) *Mitochondria*

Mitochondria represent an organelle capable of accumulating Ca^{2+}. Ca^{2+} uptake into the mitochondria does not involve a Ca^{2+} ATPase, but occurs via a uniporter (Gunter and Pfeiffer, 1990) which can operate in both directions. Ca^{2+} transport into the mitochondria is driven by an electrochemical proton gradient across the inner mitochondrial membrane. Ca^{2+} efflux from the mitochondria is mediated by different mechanisms depending on the cell type. In Ca^{2+}-flux experiments on permeabilized cells or microsomes, it is necessary to completely diminish Ca^{2+} uptake into mitochondria in order to see the effect of the ER. The most commonly used pharmacological tools for this purpose do not affect the mitochodrial uniporter directly but interfere with the generation of the proton-motive force and therefore inhibit mitochondrial Ca^{2+} uptake. Examples are sodium azide, oligomycin, antimycin and carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (an uncoupler), which are used in combination.

A significant involvement of the mitochondria in Ca^{2+} homeostasis is generally believed only to occur when the cellular [Ca^{2+}] rises to levels so high that they might become toxic to the cell (Meldolesi *et al.*, 1990). The mitochondria have a much lower affinity for Ca^{2+} than the sarco-(endo)plasmic reticulum Ca^{2+} pumps (Carafoli, 1988).

IV. REGULATION OF INTRACELLULAR CALCIUM BY THE ER

1. Morphology of the ER

ATP-driven Ca^{2+} uptake, Ca^{2+} storage, and IP_3-induced release of stored Ca^{2+} are key functions of the ER which contribute to intracellular Ca^{2+} homeostasis. The ER, however, is morphologically and functionally a

heterogeneous organelle. Although the ER as a whole is most likely a morphological continuum in all cells (Baumann and Walz, 1989), it has several morphologically and topologically different subregions (Walz and Baumann, 1989). It can be subdivided into the rough and smooth portions and the nuclear envelope. Moreover, it has been found that ER-subregions invade glycogen-rich cytoplasmic domains and ER-cisternae associate with components of the cytoskeleton of the mitochondria. The application of the Ca^{2+}-oxalate method for the localization of Ca^{2+}-squestering ER in several cell types indicates that all subregions of the ER are able to accumulate Ca^{2+} (Walz and Baumann, 1989). Evidence from biochemical studies show that the ER has IP_3-sensitive and -insensitive Ca^{2+} pools (Biden *et al.*, 1986).

2. Calcium-binding Proteins of the ER

Calsequestrin, a luminal SR protein, is considered a crucial requirement for the calcium storage function in this organelle (Fliegel *et al.*, 1987; Scott *et al.*, 1988; Milner *et al.*, 1992). Since the ER is a Ca^{2+} store in non-muscle cells, the expectation is that proteins with similar function should exist in the ER. At least five major proteins associated with the lumen of the ER have been found (Lewis *et al.*, 1985; Koch *et al.*, 1986; Macer and Koch, 1988). These include calreticulin, ERP99, BiP (immunoglobulin heavy chain binding protein), protein disulphide isomerase and RP60. One of these, calreticulin, is well characterised (Smith and Koch, 1989; Fliegel *et al.*, 1989). Like calsequestrin, calreticulin binds Ca^{2+} with a low affinity and high capacity (Milner *et al.*, 1992). Results obtained by cDNA cloning and sequencing show that skeletal muscle calsequestrin and calreticulin contain two short stretches with similar sequences (Smith and Koch, 1989). Calreticulin is also reported to be present in the SR (Fliegel *et al.*, 1989). These

intracellular Ca^{2+} binding proteins act as Ca^{2+} buffers in the lumen of the SR/ER and thereby reduce the risk of excessive changes in cellular Ca^{2+}.

3. Calcium Uptake

1) H^+-Ca^{2+} Exchange

Schulz and colleagues have suggested that in both parotid gland and exocrine pancreas, electroneutral H^+-Ca^{2+} exchange may be more important than the Ca^{2+}-ATPase in loading the IP_3-sensitive Ca^{2+} stores (Thévenod and Schulz, 1988; Thévenod *et al.*, 1989; Schulz *et al.*, 1989). In other cells, however, dissipation of transmembrane proton gradients using protonophores did not affect IP_3-stimulated Ca^{2+} mobilization (Burgess *et al.*, 1984a; Missiaen *et al.*, 1991b). Furthermore, if H^+-Ca^{2+} exchange were the major Ca^{2+} uptake mechanism, the Ca^{2+} ionophores, A23187 and ionomycin, both of which mediate H^+-Ca^{2+} exchange, should enhance loading of the IP_3-sensitive store and not, as is generally observed, empty them (Pietrobon *et al.*, 1990).

2) Calcium Pumps

i) The SERCA Gene Family

Five different isoforms of intracellular Ca^{2+}-ATPase of mammalian cells have been described (Lytton and MacLennan, 1988; Burk *et al.*, 1989; Gunteski-Hamblin *et al.*, 1988; Lytton *et al.*, 1989; Lytton *et al.*, 1991). They are referred to as the sarcoplasmic or endoplasmic reticulum calcium ATPase family (SERCA) (Table I). These isoforms are encoded by at least three alternative spliced genes SERCA 1, 2, and 3. Tissue specific expression of the SERCA family has been demonstrated : SERCA1a is expressed in adult fast-twitch skeletal muscle,

whereas SERCA1b is found in neonatal fast-twitch skeletal muscle. SERCA2a is expressed in slow-twitch skeletal and cardiac muscle, and SERCA2b is expressed ubiquitously while SERCA3 is found in non-muscle cells (Brandl *et al.*, 1987; Lytton *et al.*, 1989; Burk *et al.*, 1989). It is not clear what the exact subcellular location of these distinct isoforms is.

A structural model for the SERCA Ca^{2+} pumps has been reported (Brandl *et al.*, 1986; Green and MacLennan, 1989). There are three major domains on the Ca^{2+} pump: (a), a transmembrane domain containing the high affinity Ca^{2+} binding sites which also form the Ca^{2+} pathway, (b), a large cytosolic head containing the ATP-binding site and acceptor aspartyl residue, and (c), the stalk region which links the membranous part to the cytosolic head.

Table I

SERCA Family of Calcium Pumps

Gene	Splice	Protein sequence	Length	Tissue
SERCA1	a	NH_2-M...G-COOH	994	Adult fast muscle
SERCA1	b	NH_2-M...DPERRK -COOH	1001	Neonatal fast muscle
SERCA2	a	NH_2-M...AILE-COOH	997	Cardiac/slow muscle
SERCA2	b	NH_2-M...GKEC(*41 residues*)LLWS-COOH	1042	Ubiquitous
SERCA3		NH_2-M...KDLK-COOH	999	Various

The proposed nomenclature (Burk *et al.*, 1989) for SERCA is used. The difference between alternatively spliced isoforms is restricted to the carboxyl termini of the proteins. The tissue distribution of messenger RNA encoding each protein is

shown. SERCA1 and SERCA2a are expressed in different muscle cells, and SERCA2b is expressed ubiquitously, while SERCA3 is found in non-muscle cells.

ii) Regulation of the ER Ca^{2+} Uptake

In isolated ER from rat liver, Ca^{2+}-pumping activity is enhanced by cAMP- and CaM-dependent protein kinases (Famulski and Carafoli, 1982).

The cellular ATP/ADP ratio is approximately 10 (Corkey *et al.*, 1986) and decreases upon stimulation of cells. The steady-state $[Ca^{2+}]_i$ in permeabilized insulinoma cells changes inversely with the ATP/ADP ratio (Corkey *et al.*, 1988). The higher steady-state $[Ca^{2+}]_i$ at a low ATP/ADP ratio is due to a decrease in the Ca^{2+}-ATPase activity. ATP and ADP share binding sites on the enzyme which might explain competitive interactions between the two ligands and consequent regulation of the ATPase activity by the ATP/ADP ratio (Corkey *et al.*, 1988).

Oxalate is the anion generally used to increase Ca^{2+} uptake into the ER of non-muscle cells as a result of its permeation into the lumen and formation of an insoluble Ca^{2+} complex. It has been shown that the IP_3-sensitive Ca^{2+} pool in DDT_1MF-2 cells is more oxalate permeable than the IP_3-insensitive Ca^{2+} pool (Ghosh *et al.*, 1989). A similar observation has been made in vascular smooth muscle cells (Missiaen *et al.*, 1991a). Ca^{2+} uptake can also be regulated by precipitating anions. Phosphate, at physiological concentrations, stimulates nonmitochondrial Ca^{2+} sequestration in liver cells (Fulcery *et al.*, 1990).

iii) Inhibitors of the ER Ca^{2+} Pump

a) *Thapsigargin.* A plant-derived sesquiterpene lactone thapsigargin has previously been shown to elevate $[Ca^{2+}]_i$ in parotid acinar cells independent of IP_3 formation (Takemura *et al.*, 1989). Thapsigargin selectively inhibits the ER Ca^{2+} pump in a variety of non-muscle cells; the cardiac SR Ca^{2+} pump is only partially

blocked while the skeletal SR Ca^{2+} pump and plasma Ca^{2+} pump are not affected (Thastrup *et al.*, 1990). The progressive switch from SERCA2b (non-muscle isoform of the Ca^{2+} pump) to SERCA2a (muscle isoform of the Ca^{2+} pump) in differentiating BC_3H1 muscle cells is accompanied by a progressive decrease in thapsigargin sensitivity (De Smedt *et al.*, 1991). The observation of a selective effect of thapsigargin on the ER Ca^{2+}-ATPase has been challenged by other studies: Sagara and Inesi (1991) have shown that thapsigargin, at subnanomolar concentrations, inhibits the SR Ca^{2+} pump of rabbit skeletal muscle. Furthermore, Lytton *et al.* (1991) report that thapsigargin inhibits all of the SERCA isoforms with the same potency and, at a similar dose range, inhibits Ca^{2+} uptake and Ca^{2+}-ATPase activity of the SR isolated from fast twitch and cardiac muscle but has no effect on either plasma membrane Na^+/K^+-ATPase or Ca^{2+}-ATPase.

Thastrup *et al.*(1990) also report that thapsigargin induces the release of Ca^{2+} from rat liver microsomes apparently by inhibition of the Ca^{2+}-ATPase responsible for Ca^{2+} uptake. Thapsigargin maximally mobilizes 85% of the oxalate-loaded, A23187-releasable Ca^{2+} from rat brain microsomes (Verma *et al.*, 1990). In all systems so far investigated, thapsigargin empties the IP_3-sensitive Ca^{2+} pool. In rat hepatocytes, thapsigargin also acts on different intracellular Ca^{2+} stores including both IP_3- and GTP-sensitive pools (Thastrup *et al.*, 1990).

These studies indicate thapsigargin is a pharmacological tool that can be used to further investigate the role of both the ER and the SR Ca^{2+}-ATPases in the regulation of intracellular Ca^{2+}.

b) *2,5-Di(tert-butyl)-1,4-benzohydroquinone* (BHQ). BHQ is a potent inhibitor of the ER Ca^{2+} pump in hepatocytes. It, though, has no effect on mitochondrial Ca^{2+} fluxes or on the plasma membrane Ca^{2+} pump activity (Moore *et al.*, 1987). In AR42J cells and pancreatic acini, BHQ treatment leads to Ca^{2+} release from two

separate pools, an agonist-sensitive pool and an agonist-insensitive pool, probably by unmasking the activity of the Ca^{2+} leaking channel present (Muallem *et al.*, 1991).

c) *GTP.* GTP has been shown to inhibit the ER Ca^{2+}-ATPase in hepatocytes (Kimura *et al.*, 1990). Since a decrease in Ca^{2+} uptake results in net Ca^{2+} release, this effect of GTP was thought to be partly responsible for the GTP-mediated Ca^{2+} release from microsomes (Kimura *et al.*, 1990), though general opinion is that GTP does not act via inhibition of Ca^{2+} accumulation (Thomas, 1988). The inhibition reported by Kimura *et al.* is also difficult to explain as GTP was found to stimulate Ca^{2+} uptake in DDT_1MF-2 cells in the presence of oxalate (Ghosh *et al.*, 1989) and in permeabilized acinar cells in the absence of oxalate (Muallem and Beeker, 1989).

4. Calcium Release

1) Action of IP_3

The interaction of agonists (Berridge and Irvine, 1984; Berridge, 1987) with their receptors at the plasma membrane initiates a cascade of reactions which amplify and propagate the signal inside the cells. At an early stage, phospholipase C (PLC) is activated (Fukui *et al.*, 1988), probably via the GTP-binding protein(s) (Exton, 1986). This causes phosphatidylinositol 4,5-bisphosphate (PIP_2) to be cleaved into the second messengers, IP_3 and DAG. DAG activates PKC (Nishizuka, 1984). IP_3 generates the calcium signal by mobilizing Ca^{2+} from nonmitochondrial intracellular stores which are likely to be specialized subcompartments of the ER (Berridge and Irvine, 1984, 1989). Inositol 2,4,5-trisphosphate, inositol 4,5-bisphosphate, and inositol 1,2 cyclic 4,5-trisphosphate have also been reported to

induce the release of Ca^{2+} but are currently thought to be physiologically less relevant (Burgess *et al.*, 1984; Connolly *et al.*, 1987).

The Ca^{2+}-mobilizing action of IP_3 is thought to be terminated when the messenger becomes metabolized. There are two metabolic routes: phosphorylation by a 3-kinase and dephosphorylation by a 5-phosphatase. The former can convert IP_3 to IP_4 and the latter can hydrolyze IP_3 to inositol 1,4-bisphosphate (Connolly *et al.*, 1987).

i) *The IP_3 Receptor*

Specific high affinity IP_3-binding sites were first identified by Spät *et al.* in liver, neutrophils and adrenal cortex (Baukal *et al.*, 1985; Spät *et al.*, 1986). The purified IP_3 receptors are homotetramers of noncovalently linked subunits (Mignery and Südhof, 1990; Maeda *et al.*, 1991). From electron microscopy of negatively-stained receptors, they appear to form 4-leaf clover-like structures about 25 nm wide (Chadwick *et al.*, 1990; Maeda *et al.*, 1990). The quaternary structure of the receptor is therefore rather similar to the ryanodine receptor, the Ca^{2+} release channel of SR (Lai *et al.*, 1988). It has been suggested that each of the four receptor subunits must bind IP_3 before the Ca^{2+} channel can open (Parker and Miledi, 1989; Meyer *et al.*, 1990).

The IP_3 receptor has been purified from several tissues (Supattapone *et al.*, 1988; Chadwick *et al.*, 1990; Mourey *et al.*, 1990). The purified IP_3 receptor has been reconstituted into phospholipid vesicles and found to both bind IP_3 with appropriate affinity and mediate IP_3-stimulated Ca^{2+} flux (Ferris *et al.*, 1989). In addition, reconstitution of the receptor into planar lipid bilayers indicated IP_3 opens a large Ca^{2+} channel (26pS) which is also permeable to Na^+ ions (Maeda *et al.*, 1991). These results confirm that the purified IP_3-binding site is the IP_3 receptor and suggest that the same protein forms both the ligand recognition

domain and the Ca^{2+} channel. These techniques will allow detailed analyses of the relationship between IP_3 binding and Ca^{2+} mobilization.

An IP_3 receptor from cerebellum has been sequenced (Mignery *et al.*, 1990) and well characterized (Shears, 1991). Recently, Südhof *et al.* (1991) have demonstrated the full structure of a new type of IP_3 receptor, referred to as type 2 IP_3 receptor as opposed to the cerebellar type 1 IP_3 receptor. The presence of different types of IP_3 receptors raises the possibility that the intracellular Ca^{2+} signalling mediated by IP_3 may also be a function of the types and distributions of the IP_3 receptors (Südhof, 1991).

IP_3 binds to the N-terminal of each receptor subunit causing a large conformational change (Mignery and Südhof, 1990) which is transmitted through a long linking domain, itself a target for regulation, to the C-terminal membrane-spanning regions which together form the Ca^{2+} channel.

ii) *Antagonists*

Heparin has been reported to bind to the IP_3 receptor with high affinity (<2 μM), and thereby competitively and reversibly antagonizes the action of IP_3 (Ghosh *et al.*, 1988). Other highly sulfated polysacchrides (e.g. pentosan sulfate, polyvinyl sulfate, dextran sulfate) also inhibit IP_3 binding (O'Rourke and Feinstein, 1990; Willcocks *et al.*, 1990). Heparin and presumably the other highly sulfated antagonists, are, however, far from specific. Among the many other effects of heparin are stimulation of L-type Ca^{2+} channels (Knaus *et al.*, 1990); uncoupling of receptors from their G proteins including those which stimulate IP_3 formation (Mousli *et al.*, 1990) and inhibition of both IP_3-kinase and the IP_4-binding site. These effects severely limit the use of heparin as an IP_3 receptor antagonist in intact cells.

iii) *Regulation of the IP_3 Receptor*

The IP_3 receptor can be regulated by both cytosolic and luminal Ca^{2+}. In cerebellum, an increase in $[Ca^{2+}]_i$ similar to that found in stimulated cells (from 0.1 to 1 μM) decreases the affinity of the IP_3-binding site and the sensitivity of Ca^{2+} mobilization to IP_3 (Worley *et al.*, 1987; Joseph *et al.*, 1989). The same pattern of regulation is seen in some other tissues including pancreatic acinar cells (Engling *et al.*, 1991) and uterine and vascular smooth muscle (Suematsu *et al.*, 1985; Varney *et al.*, 1990). However, in other tissues including platelet (Brass and Joseph, 1985) and liver (Burgess *et al.*, 1984b) similar increases in $[Ca^{2+}]_i$ have very little effect on IP_3 binding and IP_3-activated Ca^{2+} mobilization.

There is evidence to suggest that the luminal free $[Ca^{2+}]$ may regulate the IP_3 receptor (Irvine, 1990). Intracellular stores replete with Ca^{2+} are more sensitive to IP_3 (Nunn and Taylor, 1991). It appears that luminal Ca^{2+} is essential before IP_3 can open the Ca^{2+} channel (Nunn and Taylor, 1991). The luminal Ca^{2+} level also has a similar stimulatory effect on the opening of the ryanodine receptor (Nelson and Nelson, 1990).

Low concentrations of ATP, its stable analogues, or ADP increase IP_3-stimulated Ca^{2+} fluxes through the purified receptor (Ferris *et al.*, 1990). Single channel recording of the smooth muscle receptors suggest that adenine nucleotides increase the open probability of the IP_3-gated channel (Ehrlich and Watras, 1988). Interestingly, Ca^{2+}-induced Ca^{2+} release mediated by ryanodine receptors is also enhanced by adenine nucleotides (Meissner, 1984).

Phosphorylation is likely to be important in the regulation of IP_3 receptor function. In liver, cAMP potentiates IP_3-stimulated Ca^{2+} mobilization (Jenkinson and Koller, 1977; Morgan *et al.*, 1984). It appears that cAMP acts via cAMP-dependent protein kinase which was found to increase both the sensitivity of the Ca^{2+} stores to IP_3 and the maximal amount of Ca^{2+} mobilized by IP_3 (Burgess *et*

al., 1991). Recently, Ferris *et al.* (1991) have found that the reconstituted IP_3 receptor can autophosphorylate.

iv) *IP_3-sensitive Stores*

The intracellular site of IP_3 action has been an area of some controversy in recent years. Accumulated evidence suggests that the mitochondria does not act as a major cytosolic Ca^{2+} regulator in cells (Burgess *et al.*, 1983; Somlyo, 1984); the ER was ascribed this role. After subcellular fractionation of smooth muscle cells, the fractions enriched in rough ER responsed to IP_3 and those enriched in smooth ER did not (Ghosh *et al.*, 1989). While these results are consistent with the suggestion that rough ER is the IP_3-sensitive Ca^{2+} store, it can not be uniformly sensitive because there is no apparent relationship between the amount of ER within a cell and its sensitivity to IP_3 (Payne *et al.*, 1988; Krause *et al.*, 1989).

In many non-muscle cells, calreticulin has been identified in small membrane bound structures, named calciosomes. The initial studies, in which some correlation was observed between IP_3 responses and calciosome markers in subcellular fractions from HL-60 cells and phagocytes, suggested that calciosomes might be the IP_3-sensitive Ca^{2+} stores (Volpe *et al.*, 1988; Krause and Campbell, 1988). However, the status of calciosomes is not clear. Calreticulin has been found within calciosomes in some cells, and within the ER of others (Meldolesi *et al.*, 1990). Moreover, immunocytochemical studies with antibodies to the IP_3 receptor specifically label structures which do not resemble calciosomes (Satoh *et al.*, 1990).

Based on this evidence, we can be sure that IP_3-sensitive Ca^{2+} stores are nonmitochondrial, but little else is certain. Perhaps the most likely conclusion is that the major IP_3-sensitive Ca^{2+} store in most cells is likely to be a specialized component of the ER.

2) Action of GTP

Although in most cells the IP_3-sensitive stores comprise only a fraction of the total cellular Ca^{2+} stores, the different Ca^{2+} pools are believed to interact. One possible form of interaction is that Ca^{2+} may pass directly from one store to another without traversing the cytosol. GTP has been shown to facilitate the transfer between intracellular Ca^{2+} stores. Dawson (1985) was the first to report that GTP (EC_{50} < 1 mM) enhanced the ability of IP_3 to release Ca^{2+} from rat liver microsomes. The synergism occurred only in the presence of a low concentration of PEG, a membrane fusogen, and could be antagonized by a non hydrolysable analog of GTP, such as GTPγS (Gill *et al.*, 1989). Subsequent studies have shown that, in some situations, GTP substantially increased the amount of Ca^{2+} released by IP_3 (Thomas, 1988; Mullaney *et al.*, 1988). In others, GTP alone caused Ca^{2+} mobilization without affecting the response to IP_3 (Ueda *et al.*, 1986; Cheuh and Gill, 1986). GTP appears to act on a Ca^{2+} store, distinct from the IP_3-sensitive store (Henne *et al.*, 1987); the size of the GTP-mobilizable Ca^{2+} pool appears to be five times larger than that of the IP_3-releasable pool. Some studies suggest that the effects of GTP on intracellular Ca^{2+} are possibly mediated by small GTP-binding proteins (Dawson and Comerford, 1989; Gill *et al.*, 1989). These GTP-binding proteins (20-25kDa) are involved in intracellular membrane trafficking (Bourne, 1988; Bourne *et al.*, 1990), and are present in GTP-responsive vesicles (Ghosh *et al.*, 1989). Since GTP and PEG are well-known membrane fusogens (Paiement *et al.*, 1987) most of their effects on intracellular calcium release might be explained by membrane fusion processes. Studies of liver microsomes, utilizing fluorescence energy transfer, light-scattering measurements and electron microscopy, have clearly demonstrated the existence of extensive GTP-induced and PEG-supported membrane fusion (Dawson *et al.*, 1987) which could cause Ca^{2+} to leak out into the cytosol. Moreover, the concentration of GTP in the cytosol of

hepatocytes was found to be around 200 μM (Akerboom *et al.*, 1979) a concentration at which both IP_3-supported Ca^{2+} release and GTP-induced membrane fusion would be subject to continuous activation. However, neither the precise proteins involved nor their detailed mechanisms of action are clear. The physiological significance of these effects of GTP are still uncertain.

3) Action of Arachidonic Acid

Arachidonic acid and certain metabolites, generated by phospholipase A_2 (PLA_2)-catalysed breakdown of phosphatidylcholine, have been shown to be involved in signal transduction (Pelech and Vance, 1989; Exton, 1990). The cellular levels of free arachidonic acid are very low since the liberated arachidonic acid is rapidly metabolized by a membrane-bound cyclooxygenase and/or a cytosolic lipooxygenase (Smith, 1989; Holtzman, 1992). The oxygenated derivatives of arachidonic acid which are biologically active are defined as eicosanoids. Among the eicosanoids are prostaglandins, including prostacyclin, thromboxanes, leukotrienes and various hydroxy acids. In most cases, the same agonists which cause the stimulation of PLC will also induce the activation of PLA_2 (Lapetina, 1986).

Arachidonic acid has been reported to elevate $[Ca^{2+}]_i$ independent of IP_3 formation (Volpi *et al.*, 1984). Studies with permeabilized pancreatic islets have shown that arachidonic acid induces the release of Ca^{2+} from the ER (Wolf *et al.*, 1986). Arachidonic acid mobilizes Ca^{2+} from the ER in permeabilized islets with a molar potency similar to that of IP_3. Half-maximal and maximal Ca^{2+} release have been observed when arachidonic acid concentrations were 2.5 and 5 μM, respectively. Ca^{2+} release was fast and reversible. In the presence of vanadate, about half of the total releasable Ca^{2+} as measured using the Ca^{2+} ionophore A23187 was mobilized from the ER by arachidonic acid (Turk *et al.*, 1987). It was

therefore proposed that in the β cells, arachidonic acid acts as a Ca^{2+}-mobilizing second messenger, similar to IP_3. In single pancreatic islet β cells, arachidonic acid (5-30 μM) has been reported to induce a biphasic rise in $[Ca^{2+}]_i$ with an early transient phase reflecting mobilization of intracellular Ca^{2+} and a later sustained phase reflecting Ca^{2+} entry from the extracellular space. These effects are stimulated by arachidonic acid itself and not its metabolite(s) (Ramanadham *et al.*, 1992). Similar results have been demonstrated using a T-lymphocyte cell line (Chow and Jondal, 1990). In addition, in both intact (Naccache *et al.*, 1989) and permeabilized neutrophils (Beaumier *et al.*, 1987), arachidonic acid has been shown to induce a release of calcium from intracellular pools. The mechanism by which arachidonic acid induces Ca^{2+} release is unknown. The simplest explanation would be that arachidonic acid increases membrane permeability by perturbation of the hydrophobic regions of the bilayer. Anionic lipids, like phosphatidic acid and anionic fatty acids, especially arachidonic acid, are known to increase membrane leakiness (Philipson and Ward, 1985). It has also been reported that arachidonate-induced Ca^{2+} efflux from platelet microsomes is mediated by an ionophoric mechanism (Fischer *et al.*, 1990).

AIMS OF THIS STUDY

The pancreatic ductal epithelium constitutes only 14% of pancreatic tissue in man (Githens, 1988), but plays an important role in bicarbonate fluid secretion. Defects in ductal function may occur in cystic fibrosis (Gaskin *et al.*, 1982; Kopelman *et al.*, 1985), and perhaps acute pancreatitis (Reber *et al.*, 1986). Since Ca^{2+} has been shown to trigger and control cell secretion in many cell systems it may be involved in the regulation of ductal secretion.

An important development in the understanding of Ca^{2+} regulation in cells was the establishment of the link between the plasma membrane receptor occupation, inositol-containing lipid hydrolysis and changes in $[Ca^{2+}]_i$. Streb *et al.* (1983) first reported that IP_3 and hormones released Ca^{2+} from the same nonmitochondrial intracellular stores of permeabilized pancreatic acinar cells. Subsequent studies confirmed that IP_3 has similar effects in countless cell types from animals and plants (Berridge and Irvine, 1984, 1989). Unfortunately, little is known about the regulation of the intracellular Ca^{2+} concentration in human pancreatic ductal cells. Therefore, the mechanisms of intracellular Ca^{2+} mobilization in this cell type are of great interest.

It is very difficult to study ductal function *in situ*, because the ductal cells comprise a small proportion of the pancreas. Therefore, biochemical studies on whole glands must largely reflect the properties of acinar cells, which form 74-85% of the tissue (Githens, 1988). Although techniques are now available for the isolation of small pancreatic ducts (Argent and Gray, 1990) and monolayer culture of functional pancreatic ductal epithelia (Harris, 1990), they have only been applied to copper deficient rat pancreas (Argent and Gray, 1990) and human fetal pancreas (Harris, 1990). A PANC-1 cell line (ductal cells derived from a human pancreatic carcinoma) has been reported to maintain some biochemical and

morphological characteristics of normal ductal epithelium (Madden and Sarras, Jr., 1988), and they are easy to grow. This cell line was used as a model system for this study.

The following are the aims of this study:

1) Using microsomes prepared from PANC-1 cells to identify and characterize intracellular Ca^{2+} pools.

2) Using the microsomes to investigate the mechanisms of intracellular Ca^{2+} movement as well as possible interaction between different releasable Ca^{2+} pools.

It is hoped that the information obtained from this study will contribute to our understanding of intracellular Ca^{2+} regulation in pancreatic ductal cells.

MATERIALS AND METHODS

I. MATERIALS

The chemicals were obtained from the following companies:

(All reagents were analytical grade.)

BDH Chemicals Canada Ltd.

- Calcium chloride
- Chloroform
- Folin & Ciocalteu reagent
- Hydrochloric acid
- Magnesium chloride
- Potassium chloride
- Sodium carbonate
- Sodium hydroxide
- Sucrose

Boehringer Mannheim Canada Ltd.

- Bovine serum albumin
- D-*myo*-Inositol 1,4,5-trisphosphate
- Dithiothreitol
- Phenylmethylsulfonyl fluoride

Calbiochem Corporation

- A23187

Hyclone

- Dulbecco's modified Eagle's medium
- Fetal bovine serum

ICN Radiochemicals

- $^{45}CaCl_2$ (5-30 mCi/mg calcium)

LC Services Corporation

Thapsigargin

Mallinckrodt

Copper sulphate

Sodium tartrate

Serdary Research Laboratories Inc.

Arachidic acid

Arachidonic acid

Elaidic acid

Linoleic acid

Palmitoleic acid

Stearic acid

Sigma Chemical Company

Adenosine 5'-triphosphate

Benzamidine

EGTA

GTP

GTPγS

Heparin (MW: 4,000-6,000)

Imidazole

Indomethacin

Nordihydroguaiaretic acid

Oligomycin

Penicillin-G

Polyethylene glycol (MW: 8,000)

Sodium azide

Sodium deoxycholate

Streptomycin sulphate

Trichloroacetic acid (crystalline)

II. METHODS

1. Culture Conditions

PANC-1 cells were plated on 175 cm^2 plastic tissue culture flasks at a density of $1x10^7$ cells/flask and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, $6.0x10^4$ U/l Penicillin and $4.5x10^4$ U/l Streptomycin. The cells were maintained at 37°C in a humidified 5% CO_2 air mixture. The cells reached confluency within 6-7 days. Cells were passaged using trypsin digestion.

2. Preparation of PANC-1 Microsomes Enriched in the ER

Microsomes were prepared from PANC-1 cells as follows: cultured cells were harvested in buffer containing 25 mM imidazole, pH 7.4, 0.25 M sucrose, 1 mM benzamidine, 5 mM dithiothreitol (DTT) and 0.1 mM phenylmethyl-sulfonylfluoride (PMSF) at 4°C. Cells were centrifuged at 100 xg for 10 min. Cell pellets were resuspended in the same buffer (approximately 1 volume of cells in 10 volumes of buffer) and homogenized with a motor driven Potter-Elvehjem homogenizer (15 strokes) followed by a glass Dounce homogenizer (15 strokes). The homogenate was centrifuged at 300 xg for 8 min to remove large cell debris and unbroken cells and the supernatant was saved. The pellet was resuspended in a similar volume of buffer and homogenization was repeated. The saved supernatant was combined with new homogenate and centrifuged at 2,000 xg for 8 min. Following two centrifugations (9,500 xg for 10 min; 100,000 xg for 30 min) the pellet was resuspended in calcium uptake buffer (see below), quickly frozen in

liquid nitrogen, and stored at -80°C. The subcellular fractions were enriched by approximately 3-fold in ER as determined by the distribution of the marker enzyme, NADPH cytochrome c reductase.

3. Measurement of $^{45}Ca^{2+}$ Uptake and Release Activities

For $^{45}Ca^{2+}$ uptake, PANC-1 microsomes were preincubated at 37°C at a protein concentration of 350 μg/ml in calcium uptake buffer containing: 100 mM imidazole, pH 7.0, 6.0 mM $MgCl_2$, 2.0 mM NaN_3, 100 mM KCl, 0.25 M sucrose, 10 μg/ml oligomycin (inhibitor of mitochondrial Ca^{2+} uptake), 0.195 mM $CaCl_2$ (0.1 μM free calcium) containing $^{45}CaCl_2$ (with 0.75 Ci/mole $^{45}Ca^{2+}$), 1.05 mM EGTA. An aliquot (100 μl) was taken for determination of passive uptake after 4 min. ATP (5.72 mM) was added to induce active Ca^{2+} accumulation. Calcium release from the microsomes was measured following the addition of different Ca^{2+} mediators: IP_3, GTP, arachidonic acid and thapsigargin. Samples were taken at different time points, filtered through 0.45 μM pore size Millipore filters and washed within 30 sec with ice cold uptake buffer. $^{45}Ca^{2+}$ uptake was quantitated by liquid scintillation counting.

The calcium uptake by PANC-1 microsomes, expressed in nanomoles calcium transported per mg protein, was calculated by using the following formula:

$$\frac{\text{(sample counts-blank counts) x total calcium}}{\text{(total counts-blank counts) x mg protein}}$$

where:

sample counts = ^{45}Ca counts (cpm) obtained per 100 μl

sample

total counts = total ^{45}Ca counts (cpm) in 100 µl of uptake medium

blank counts = counts (cpm) obtained in the presence of scintillation counting medium alone

total calcium = total amount of calcium present in 100 µl of uptake medium (= 19.5 µmoles)

mg protein = amount of microsomal protein present in the 100 µl of uptake medium

4. Preparation and Use of Phospholipid Vesicles

Large unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) were prepared by extrusion using the method of Hope *et al.* (1985), in calcium uptake buffer (see above) containing 10 µCi/ml $^{45}Ca^{2+}$ and 1 µM unlabelled Ca^{2+}. Vesicles were incubated at 37°C for up to 1 hour in the presence of 100 µM arachidonic acid or 100 nM thapsigargin or 1 µM A23187 as a positive control. Unentrapped calcium was removed by passing the vesicles down a 10 ml Sephadex G-50 column. Vesicles entrapped calcium were counted by a liquid scintillation counter.

5. Protein Determination

Protein concentration is commonly determined by the method of Lowry *et al.* (1951). However, DTT interfered with the assay by increasing the blank (Bensadoun and Weinstein, 1976). Samples were prepared following the method of Bensadoun and Weinstein (1976) to remove the interference. Proteins are solubilized and precipitated with trichloroacetic acid leaving the interfering substance in the supernatant.

Samples were assayed in 1.2 ml medium containing 0.025% sodium deoxycholate and 8% trichloroacetic acid, and left on ice for 10 min. The resulting precipitate was centrifuged at 8,000 xg for 2 min in an Eppendorf microfuge and the pellet was assayed for protein.

One ml of Lowry reagent A/B was added, (where A = 2% Na_2CO_3 in 0.1 N NaOH, 0.04% sodium tartrate; B = 1% copper sulphate, mixed in a 100:2 ratio v/v), mixed and left at room temperature for 10 min. To this, 100 µl of Folin Ciocalteu reagent (diluted 1:1, v/v, in ddH_2O) was added, the contents were mixed and left for 30 min before measuring the absorbance at 750 nm. The sample absorbance was compared to bovine serum albumin standards and the assay was found to be linear to 80 µg.

6. Determination of Free Ca^{2+} Concentrations

Free calcium concentrations were determined by the method of Fabiato (1988).

RESULTS

Using microsomes enriched in the ER prepared from PANC-1 cells, an investigation of the Ca^{2+} accumulation and release properties was conducted.

I. Ca^{2+} ACCUMULATION BY PANC-1 MICROSOMES

$^{45}Ca^{2+}$ was actively accumulated into PANC-1 microsomes in a ATP-dependent manner. The initial rate of Ca^{2+} uptake was a function of the free Ca^{2+} concentration in the uptake medium (Fig.1). The half-maximal activation of Ca^{2+} occurred at approximately 0.29 ± 0.02 μM (n = 5) free Ca^{2+}. The maximal initial rate of Ca^{2+} uptake (V_{max}) was 15.3 ± 0.3 nmol/mg/min (n = 5). Following the addition of ATP, ATP-dependent Ca^{2+} accumulation reached a steady state after 5 min and the amount of accumulated Ca^{2+} remained unchanged for 30 min (Fig.2). In subsequent experiments, five min was taken as the time point for measuring active Ca^{2+} uptake.

II. IP_3-MEDIATED CALCIUM RELEASE

1. Concentration-dependent Effect of IP_3 on the Release of Ca^{2+} from PANC-1 Microsomes

As shown in Fig. 3, IP_3 stimulated the release of pre-loaded $^{45}Ca^{2+}$ from PANC-1 microsomes in a concentration-dependent manner with an EC_{50} of approximately 0.35 μM. Maximally, IP_3 (3 μM) released approximately 20% of the actively accumulated Ca^{2+}. IP_3-induced Ca^{2+} release was fast; the addition of IP_3 led to a release of Ca^{2+} within 30 sec. The released Ca^{2+} was reaccumulated into the microsomes within 5 minutes (Fig. 4).

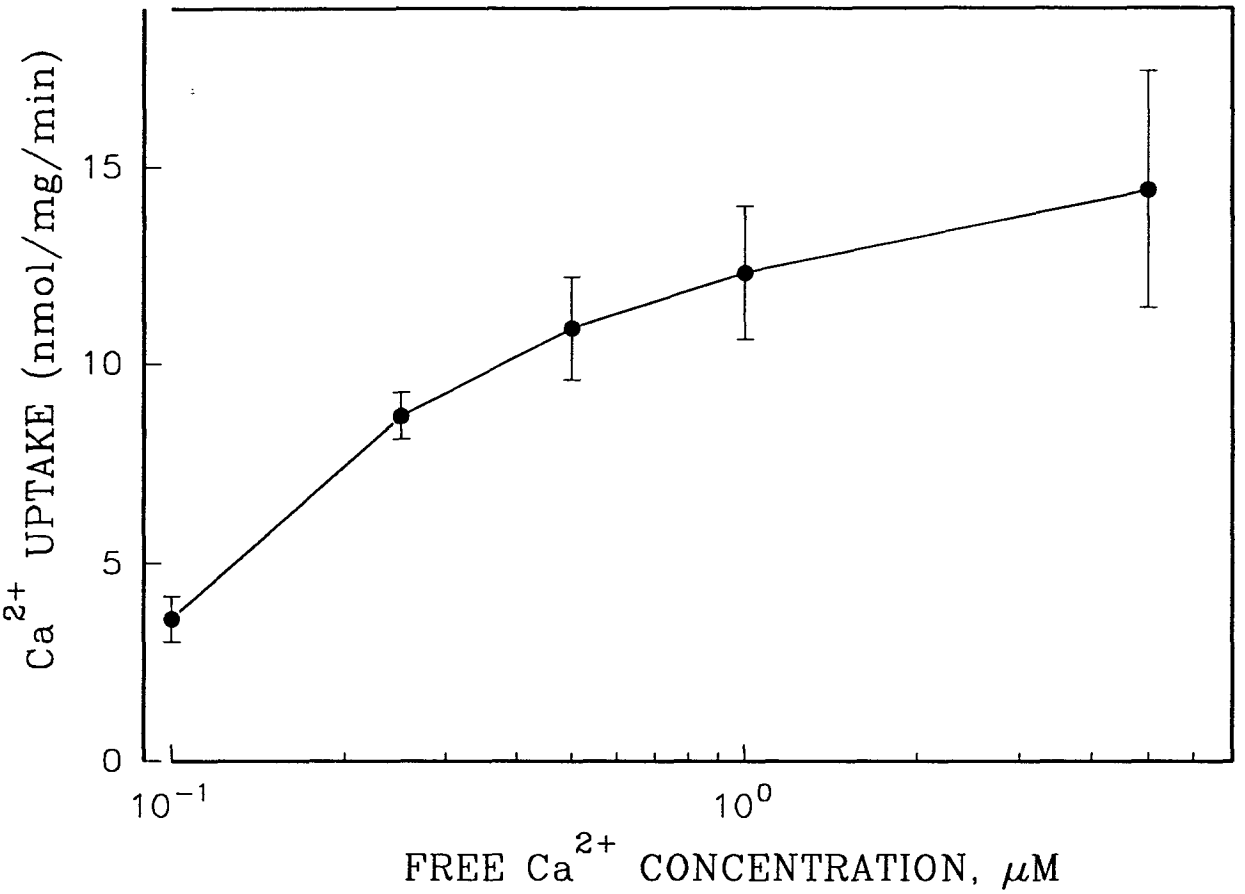

Fig. 1. Calcium uptake as a function of free calcium concentration. PANC-1 microsomes were preincubated in the uptake medium containing different free Ca^{2+} concentrations. Ca^{2+} uptake was determined at 20 sec after the addition of ATP. Results represent the mean of 5 separate experiments (mean ± S.E.).

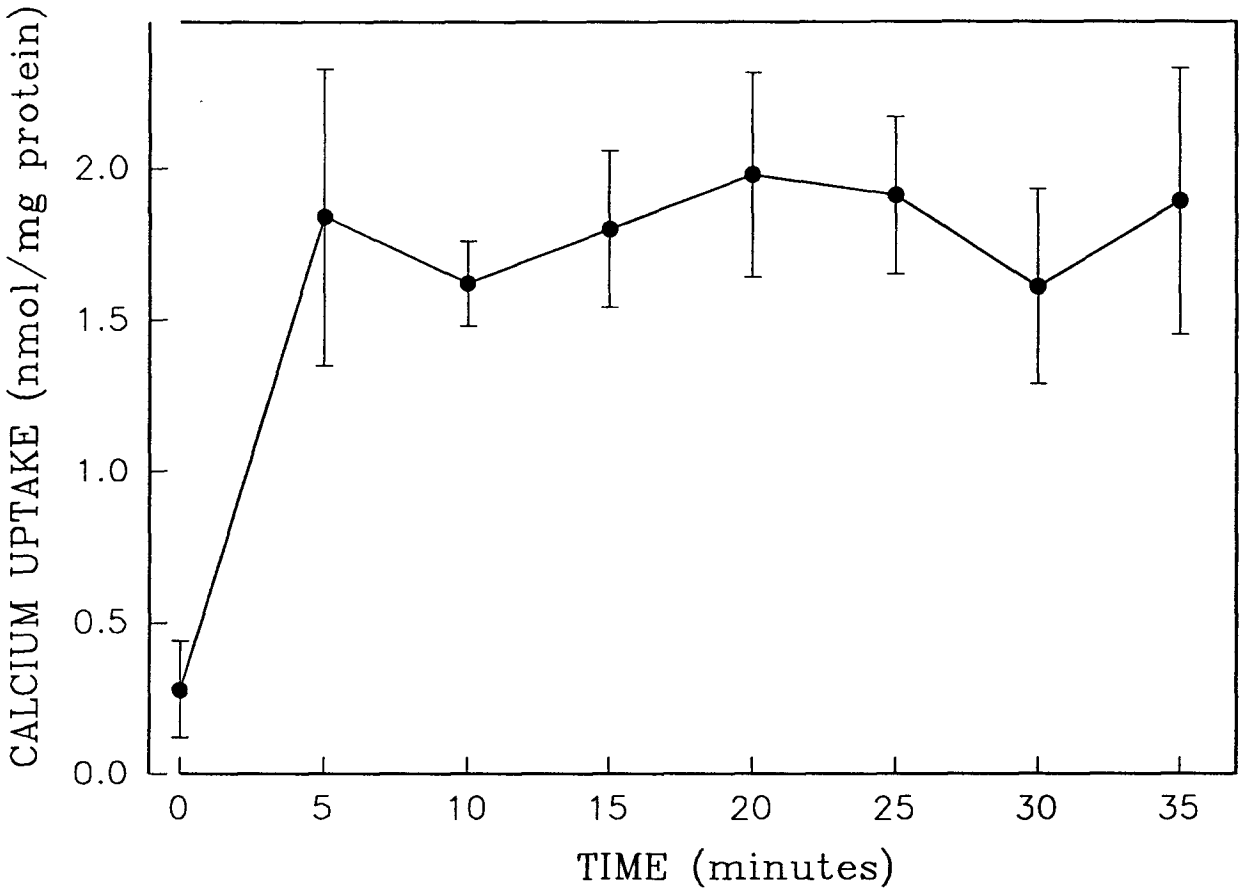

Fig. 2. ATP-activated calcium accumulation by microsomes isolated from PANC-1 cells. The microsomes were preincubated in the uptake medium for 5 min. Following the addition of ATP, Ca^{2+} uptake was measured at 5, 10, 15, 20, 25, 30 and 35 min. Results represent the mean of 3 separate experiments (mean ± S.E.).

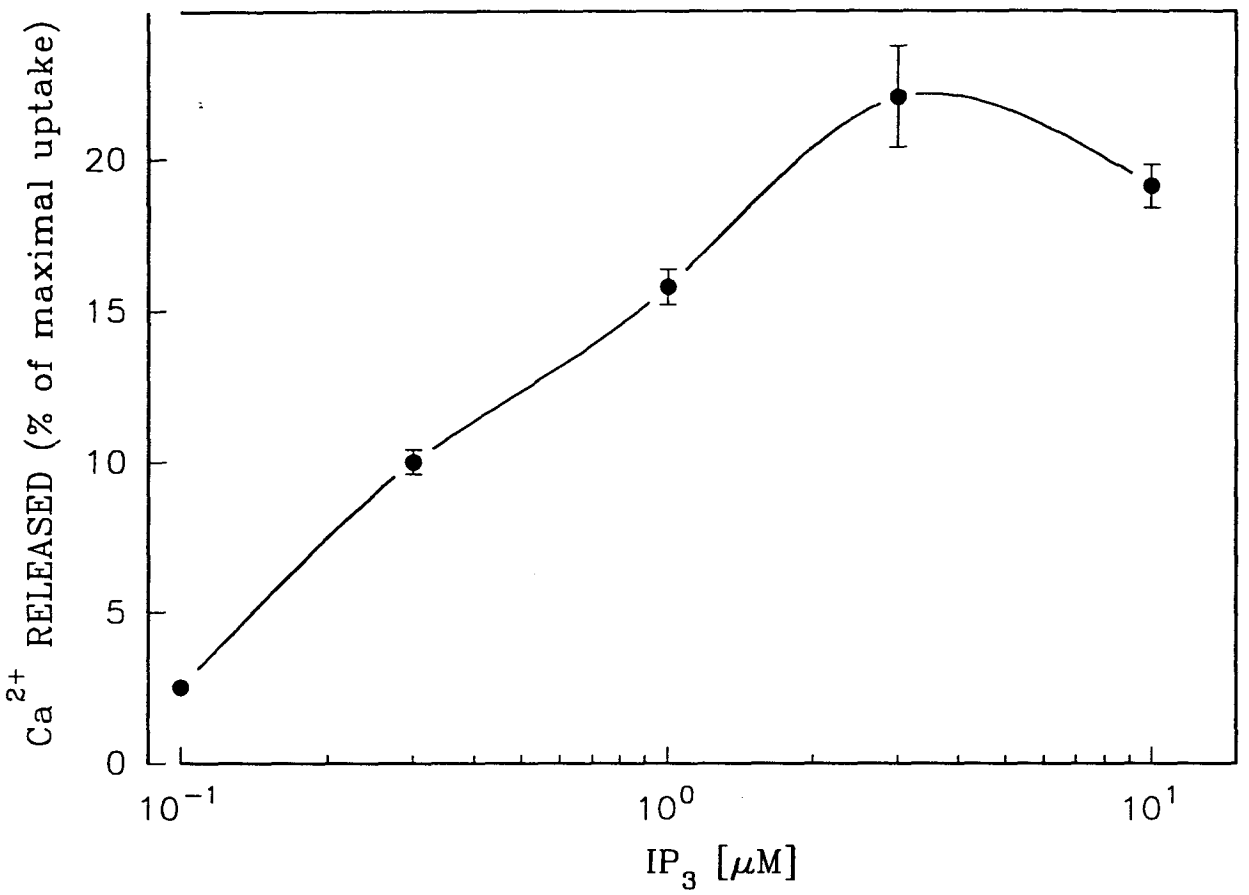

Fig. 3. Concentration-dependent effect of IP_3 on the release of Ca^{2+} from PANC-1 microsomes. The microsomes were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. IP_3-induced Ca^{2+} release was determined at 30 sec following addition of IP_3. Results represent the mean of 5 separate experiments (mean ± S.E.).

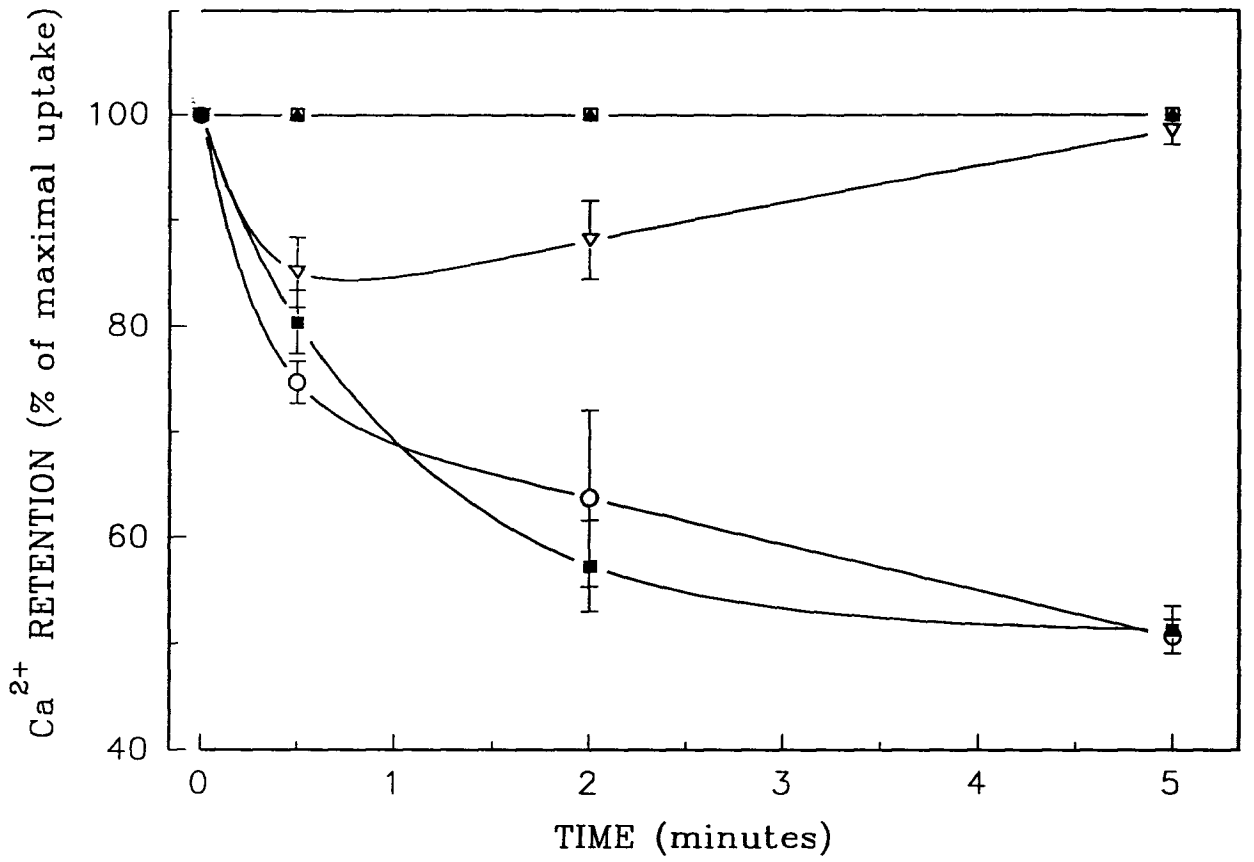

Fig. 4. Effects of heparin on IP_3- or GTP-mediated Ca^{2+} release from PANC-1 microsomes. The microsomes were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. Ca^{2+} release was determined in the presence of 1 μM IP_3 (▽), 100 μM GTP and 3% (w/v) PEG (■), 30 μg/ml heparin and 1 μM IP_3 (▲), 30 μg/ml heparin, 100 μM GTP and 3% (w/v) PEG (○), and ddH_2O (❑). Heparin was added 2 min before the addition of IP_3 or GTP. The data shown are the mean of 3 separate experiments (mean ± S.E.).

2. Effect of Extravesicular Ca^{2+} Concentration on IP_3-mediated Ca^{2+} Release

The amount of Ca^{2+} released in response to IP_3 was decreased with increasing free Ca^{2+} concentrations in the uptake medium (Fig. 5). Ca^{2+} uptake and release were normally measured at a free Ca^{2+} concentration of 0.1 μM. When the free Ca^{2+} concentration was increased to 0.58 μM, the effect of IP_3 was reduced by 50%. At 1.0 μM free Ca^{2+}, the effect was completely abolished.

3. Effect of Heparin on IP_3-mediated Ca^{2+} Release

Addition of heparin (30 μg/ml), a competitive antagonist of the IP_3 receptor, 2 min before addition of IP_3 completely inhibited IP_3-induced Ca^{2+} release (Fig. 4).

III. GTP-INDUCED Ca^{2+} RELEASE

1. Effect of PEG on GTP-induced Ca^{2+} Release

As shown in Table II, GTP by itself induced only 5% release of the accumulated Ca^{2+} from PANC-1 microsomes. PEG (3%, w/v) alone had no effect on Ca^{2+} mobilization but significantly enhanced the effectiveness of GTP in inducing Ca^{2+} release (62%). PEG (3%, w/v) was therefore included in the Ca^{2+} transport medium in order to allow further study of the effect of GTP on Ca^{2+} release.

2. Concentration-dependent Effect of GTP on the Release of Ca^{2+}

Fig. 6 shows the concentration-dependent effect of GTP on Ca^{2+} release: maximally, GTP at 100 μM induced the release of 62.4 ± 5% (n = 3) of the accumulated Ca^{2+} from PANC-1 microsomes. Thus, the GTP-mobilizable Ca^{2+} pool was approximately three times larger than the IP_3-sensitive pool (20%).

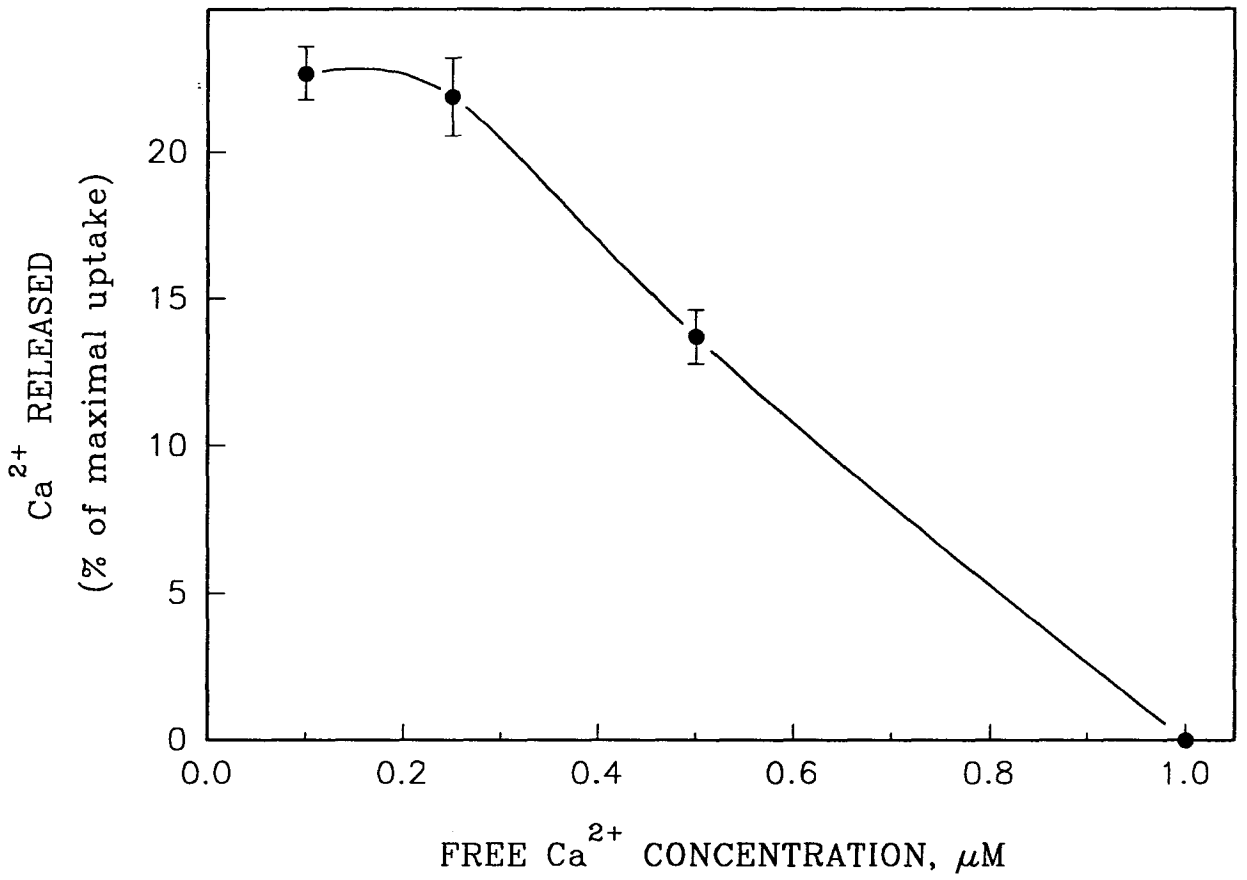

Fig. 5. Effect of extracellular Ca^{2+} concentration on IP_3-induced Ca^{2+} release from PANC-1 microsomes. The microsomes were preincubated in the uptake medium containing different free Ca^{2+} concentrations. The release of the preloaded Ca^{2+} was carried out with 1 μM IP_3 and determined at 30 sec following the addition of IP_3. Results represent the mean of 3 separate experiments (mean ± S.E.).

Table II

Effects of GTP and IP_3 on the Release of the Preloaded Ca^{2+} from PANC-1 Microsomes

Treatment	Ca^{2+} Released (% of maximal uptake)	
100 μM GTP (5 min)	4.8±0.6	(n=6)
3% PEG (5 min)	0	(n=3)
100 μM GTP + 3% PEG (5 min)	62.4±5.0	(n=3)
100 μM GTPγS + 3% PEG (5 min)	0	(n=3)
1 μM IP_3 (30 sec)	15.3±0.58	(n=5)
1 μM IP_3 + 3% PEG (30 sec)	14.9±1.5	(n=3)
100 μM GTP + 3% PEG (30 sec)	37.4±2.1	(n=5)
1 μM IP_3 + 100 μM GTP + 3% PEG (30 sec)	46.7±6.4	(n=6)

Calcium release was measured as described in Materials and Methods. PANC-1 microsomes were preincubated for 5 min in the uptake medium in the presence or the absence of 3% (w/v) PEG. Following addition of ATP, Ca^{2+} was preloaded into the microsomes. Ca^{2+} released was determined at 30 sec or 5 min. Values are means ± S.E. of separate experiments (n).

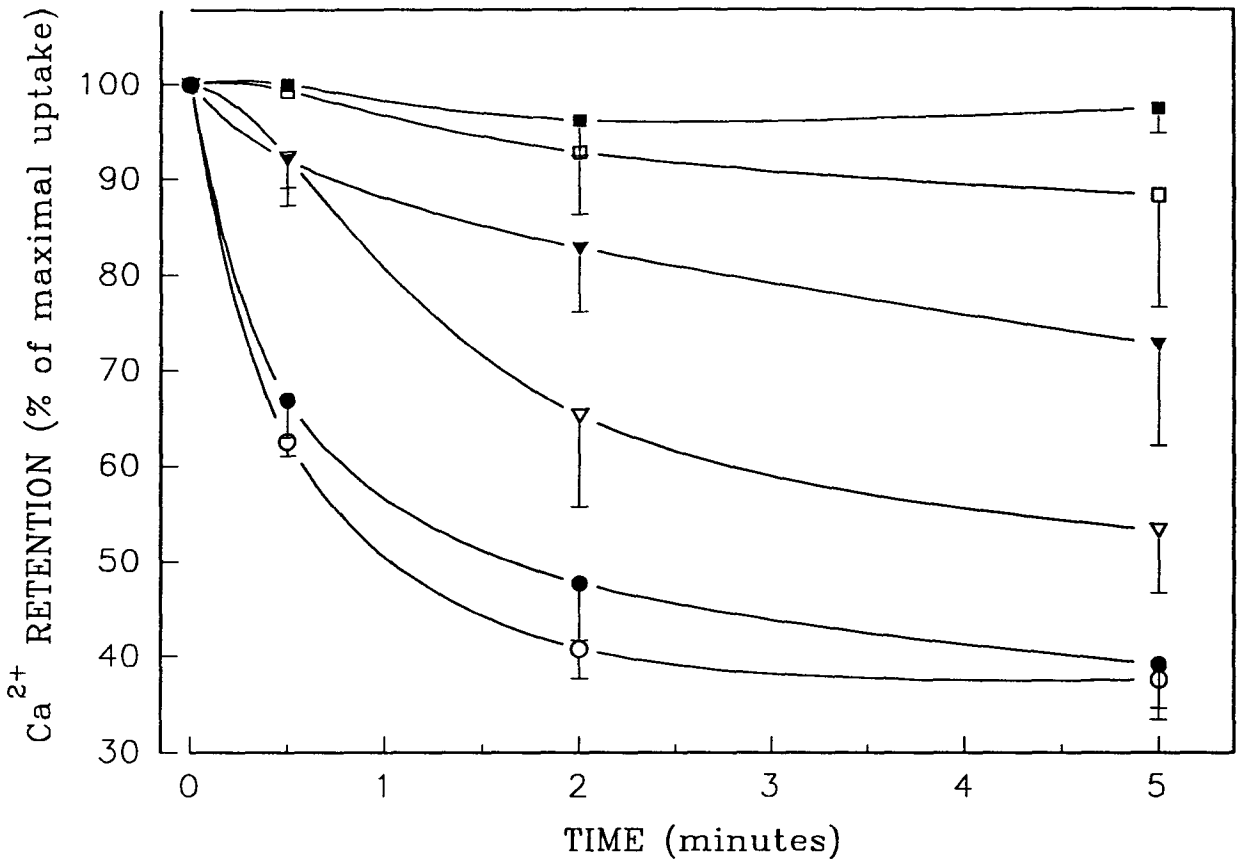

Fig. 6. Time- and concentration-dependent effects of GTP on the release of the accumulated Ca^{2+} from PANC-1 microsomes. The microsomes were preincubated in the uptake medium containing 3% (w/v) PEG and preloaded with $^{45}Ca^{2+}$ in the presence of ATP. After a maximal uptake of Ca^{2+}, GTP was added to a final concentration of 0 (■), 1 (❑), 3 (▼), 10 (▽), 30 (●) and 100 (○) μM. Ca^{2+} release was determined at 0.5, 2 and 5 min. Results represent the mean of 3 separate experiments (mean ± S.E.).

Kinetically unlike IP_3, GTP-induced Ca^{2+} release was slower in reaching its maximal effect.

3. Combined Effects of GTP and IP_3 on Ca^{2+} Release

As discussed previously, IP_3 induced a maximal Ca^{2+} release at 30 sec and the Ca^{2+} was quickly reaccumulated by the microsomes (Fig. 4). In order to examine the combined effects of GTP and IP_3, it was necessary to take measurements at the 30 sec time point. When GTP (in the presence of PEG) was added simultaneously with IP_3, the Ca^{2+} release at 30 sec was larger than either agent alone indicating GTP and IP_3 mobilize different Ca^{2+} pools (Table II). Moreover, GTP-induced Ca^{2+} release was completely heparin-insensitive (Fig. 4). These results suggest that GTP and IP_3 induce Ca^{2+} release from PANC-1 microsomes via different mechanisms.

4. Effect of GTPγS on the Release of Ca^{2+}

Addition of GTPγS, a non-hydrolyzable analogue of GTP, did not induce any Ca^{2+} release in the presence of PEG (Table II), indicating that the action of GTP may involve a GTP hydrolytic process.

IV. ARACHIDONIC ACID-MEDIATED Ca^{2+} RELEASE

1. Concentration-dependent Effect of Arachidonic Acid on the Release of Ca^{2+}

Addition of arachidonic acid to the microsome preparations induced a rapid release of the accumulated $^{45}Ca^{2+}$. Fig. 7 shows the concentration-dependent effect of arachidonic acid on the release of Ca^{2+}; the half-maximal effect was observed at approximately 45 μM and the maximal effect at approximately 100 μM

arachidonic acid. Maximally, arachidonic acid caused a rapid release of approximately 80% of the accumulated Ca^{2+}. Thus, the arachidonic acid-releasable pool was approximately four times larger than the IP_3-sensitive pool.

2. Overlap of the Arachidonic acid- and IP_3-releasable Ca^{2+} Pools

Following sub-maximal Ca^{2+} release by arachidonic acid (50 μM), IP_3 caused a further 20% Ca^{2+} release (Fig. 8). However, as shown in Fig. 9, IP_3 was ineffective in producing any further release of Ca^{2+} from the microsomes following maximal Ca^{2+} release by arachidonic acid (100 μM). Moreover, following maximal Ca^{2+} release in response to IP_3, arachidonic acid (100 μM) could induce a further release of Ca^{2+} down to the level induced by arachidonic acid (100 μM) alone.

3. Effects of Inhibitors of Arachidonic Acid Metabolism on Arachidonic Acid-induced Ca^{2+} Release

Arachidonic acid can be rapidly metabolized by cyclooxygenase and lipoxygenase. These enzymes, especially the membrane-bound cyclooxygenase which is found in the ER (Holtzman, 1992), may be active in our microsomal preparations. A possible involvement of metabolites of arachidonic acid in the release of Ca^{2+} was investigated. The PANC-1 microsomes were pretreated with either indomethacin (20 μM, a cyclooxygenase inhibitor) or NDGA (20 μM, a lipoxygenase inhibitor) at concentrations previously shown to inhibit the metabolism of arachidonic acid in other cell types (Moncada *et al.*, 1976; Milvae and Hansel, 1983; Milvae *et al.*, 1986). As shown in Table III, indomethacin and NDGA failed to supress the arachidonic acid-activated effect, indicating that arachidonic acid exerts a direct effect on the release of Ca^{2+}.

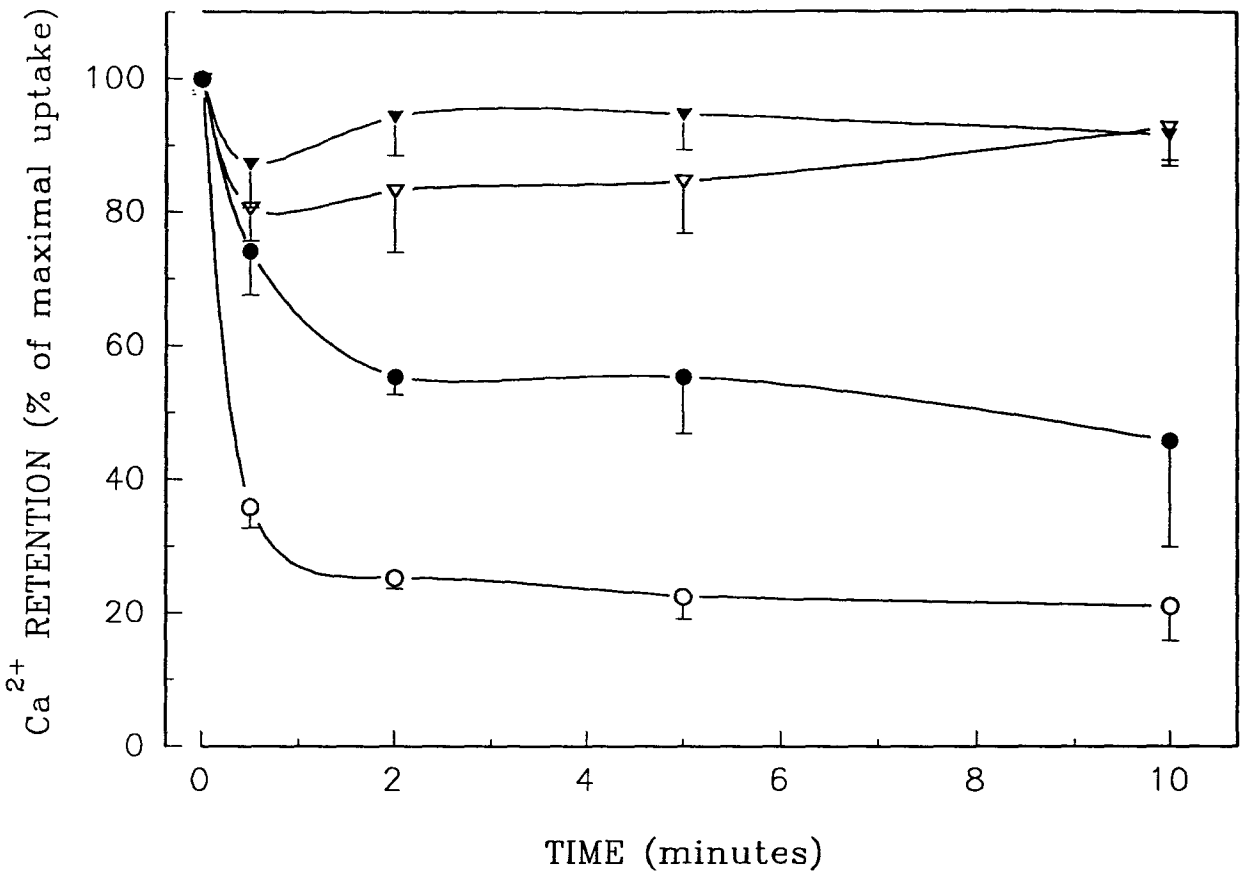

Fig. 7. Concentration-dependent effect of arachidonic acid on Ca^{2+} release from PANC-1 microsomes. The microsomes were preloaded with $^{45}Ca^{2+}$ in the presence of ATP. After a maximal uptake of Ca^{2+}, arachidonic acid was added to a final concentration of 0 (▼), 10 (▽), 50 (●), 100 (○) μM. Ca^{2+} release was measured at 0.5, 2, 5, 10 min. Results represent the mean of 3 separate experiments (mean ± S.E.).

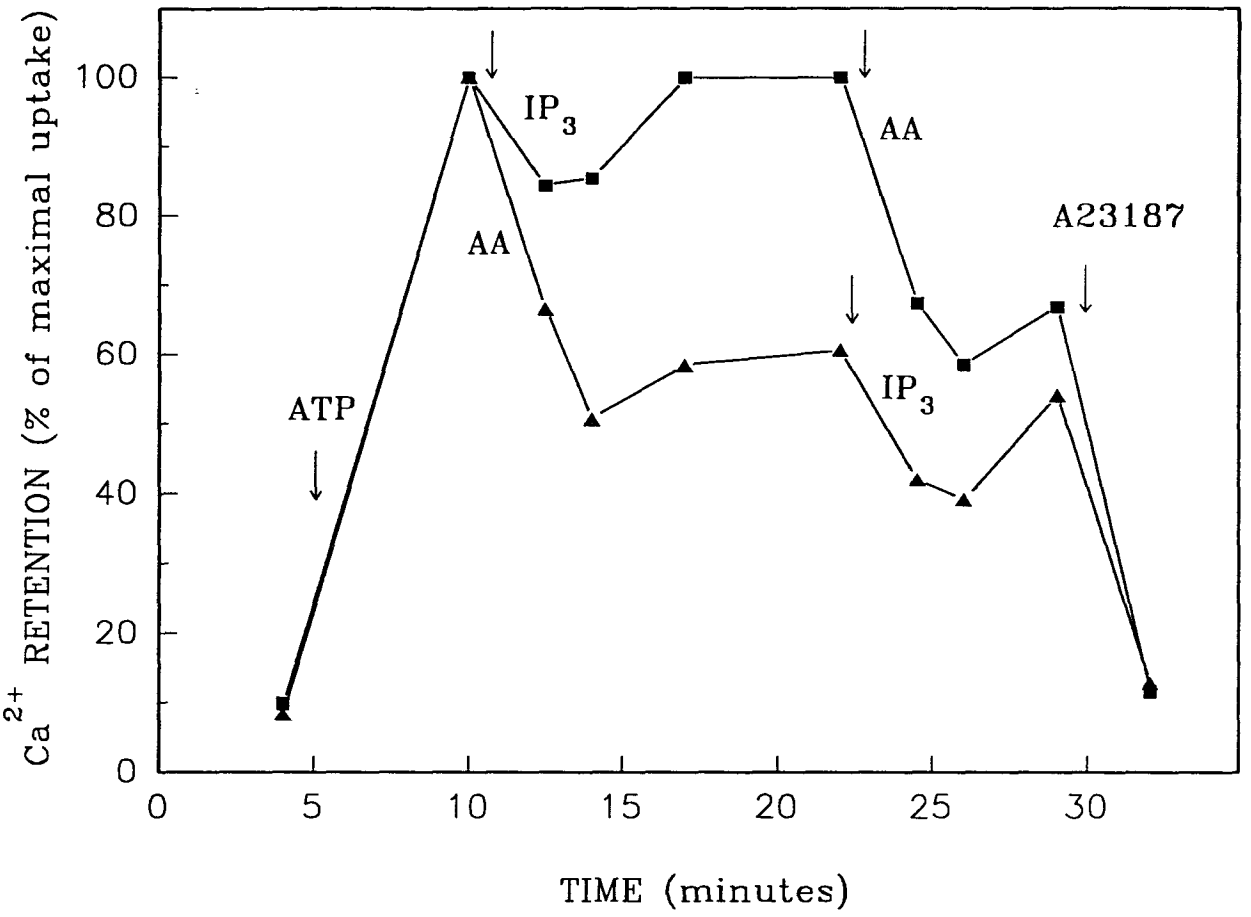

Fig. 8. IP_3- and arachidonic acid (AA)-induced Ca^{2+} release from PANC-1 microsomes. The microsomes were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. After a maximal uptake of Ca^{2+}, IP_3 (1 μM) (■) or 50 μM AA (▲) was added. Following the addition, 50 μM AA (■) or 1 μM IP_3 (▲) was subsequently applied. At the end of the experiment the action of A23187 (5 μM) was also measured. The data shown are the mean of duplicate determinations from two separate experiments.

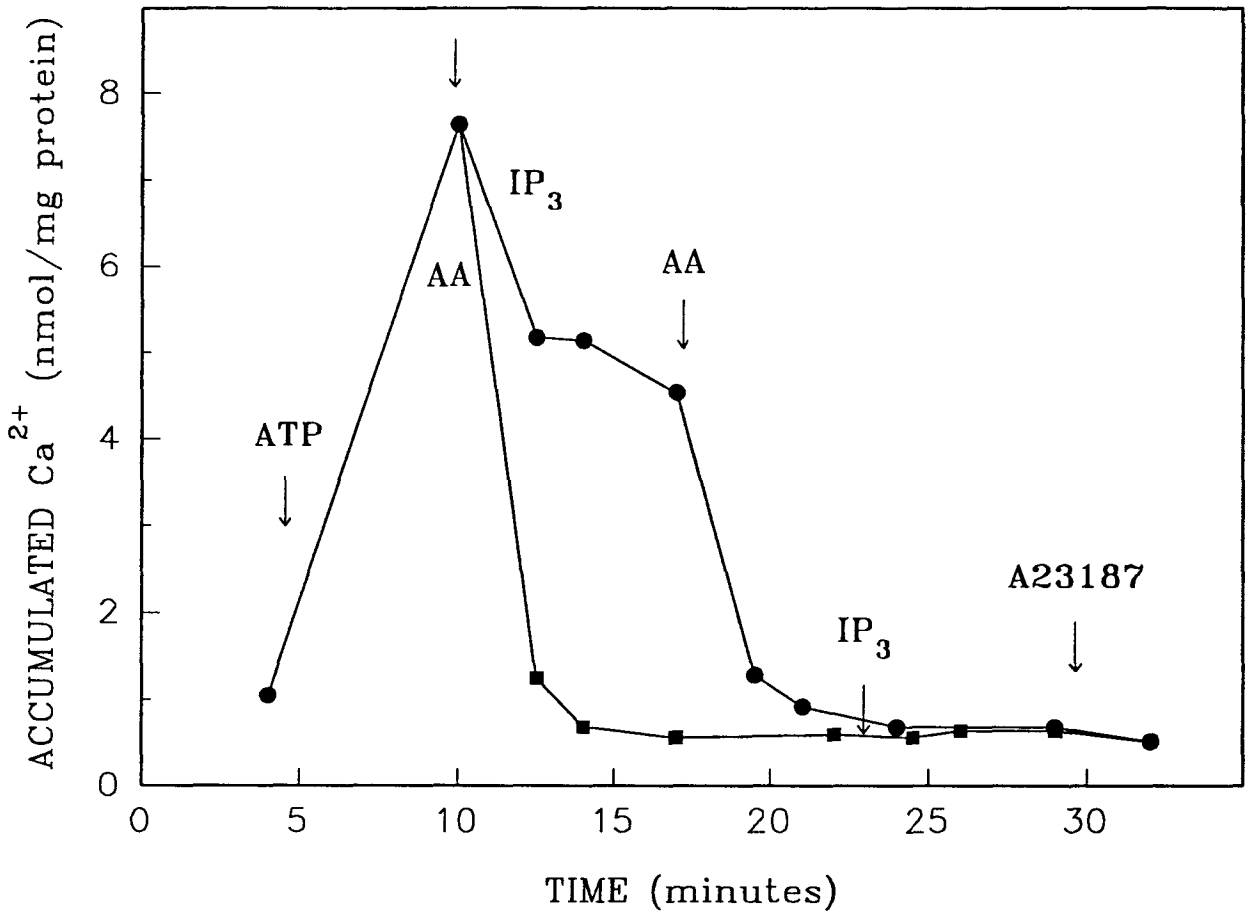

Fig. 9. Depletion of the IP_3-mediated Ca^{2+} pool by arachidonic acid (AA). The PANC-1 microsomes were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. After a maximal uptake of Ca^{2+}, IP_3 (1 μM) (●) or 100 μM AA (■) was added. Following the addition, 100 μM AA (●) or 1 μM IP_3 (■) was subsequently added. The action of A23187 (5 μM) was examined near the end of the experiment. Data represent a typical experiment.

Table III

Effects of Inhibitors of Arachidonic Acid Metabolism on Arachidonic Acid-induced Ca^{2+} Release from PANC-1 Microsomes

Treatment	Ca^{2+} Released (% of maximal uptake)
100 μM AA	80.8±1.1
20 μM Indomethacin + 100 μM AA	71.6±2.9
20 μM NDGA + 100 μM AA	74.0±5.9

Calcium release was determined as described in Materials and Methods. PANC-1 microsomes were pretreated with either indomethacin or NDGA for 2 min before the addition of arachidonic acid (AA). Following addition of AA Ca^{2+} release was measured at 20 min. Results represent the mean of 3 separate experiments (mean ± S.E.).

4. Effects of Other Fatty Acids on the Release of Ca^{2+}

The specificity of the effect of arachidonic acid on Ca^{2+} release was examined by measuring Ca^{2+} release in response to other fatty acids. As shown in Fig. 10., linoleic acid and elaidic acid induced significant Ca^{2+} release at 100 μM. Arachidic acid, palmitoleic acid as well as stearic acid, at the same concentrations, also induced Ca^{2+} release, but to a lesser extent (Fig. 10). This implies that the effect of arachidonic acid was not highly specific.

V. INHIBITION OF Ca^{2+} ACCUMULATION AND INDUCTION OF Ca^{2+} RELEASE BY THAPSIGARGIN

1. Inhibition of Ca^{2+} Uptake by Thapsigargin

Microsomes from PANC-1 cells were pretreated with thapsigargin for 5 min. This treatment induced a profound inhibitory effect on the ATP-driven accumulation of $^{45}Ca^{2+}$, as shown in Fig. 11. Half-maximal inhibition of Ca^{2+} uptake occurred at approximately 6 nM thapsigargin and maximal inhibition at 100 nM thapsigargin. Thapsigargin (100 nM) maximally inhibited 85% of the total Ca^{2+} accumulation whereas the Ca^{2+} ionophore A23187 (5 μM) blocked almost 100% of Ca^{2+} uptake (Fig.11).

2. A Depletion of the IP_3- or Arachidonic Acid-sensitive Ca^{2+} Pool by Thapsigargin

Following maximal Ca^{2+} uptake, thapsigargin induced a rapid release of approximately 85% of the total accumulated Ca^{2+}, whereas A23187 depleted 100% of actively accumulated Ca^{2+} from the microsomes (Fig. 12). When IP_3 (Fig. 12) and arachidonic acid (Fig. 13) were added after the maximal effect of thapsigargin, there was no additional release of the accumulated Ca^{2+}. The data indicate

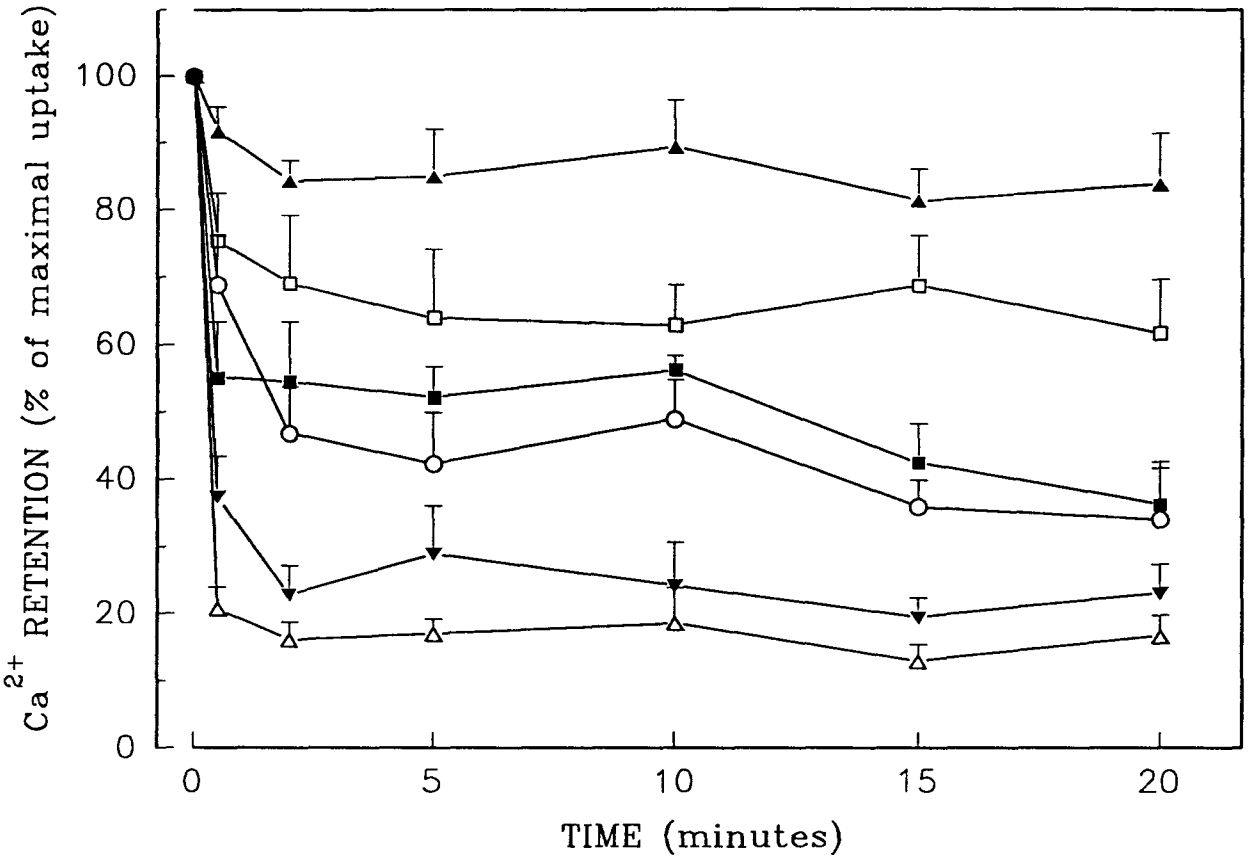

Fig. 10. Effects of other fatty acids on the release of Ca^{2+} from PANC-1 microsomes. At a concentration of 100 μM, stearic acid- (❑), palmitoleic acid- (■), arachidic acid- (○), elaidic acid- (▼), and linoleic acid- (Δ) induced Ca^{2+} release was determined at 0.5, 2, 5, 10, 15, 20 min. Ethanol was added as a solvent control (▲). Results represent the mean of 4 separate experiments (mean ± S.E.).

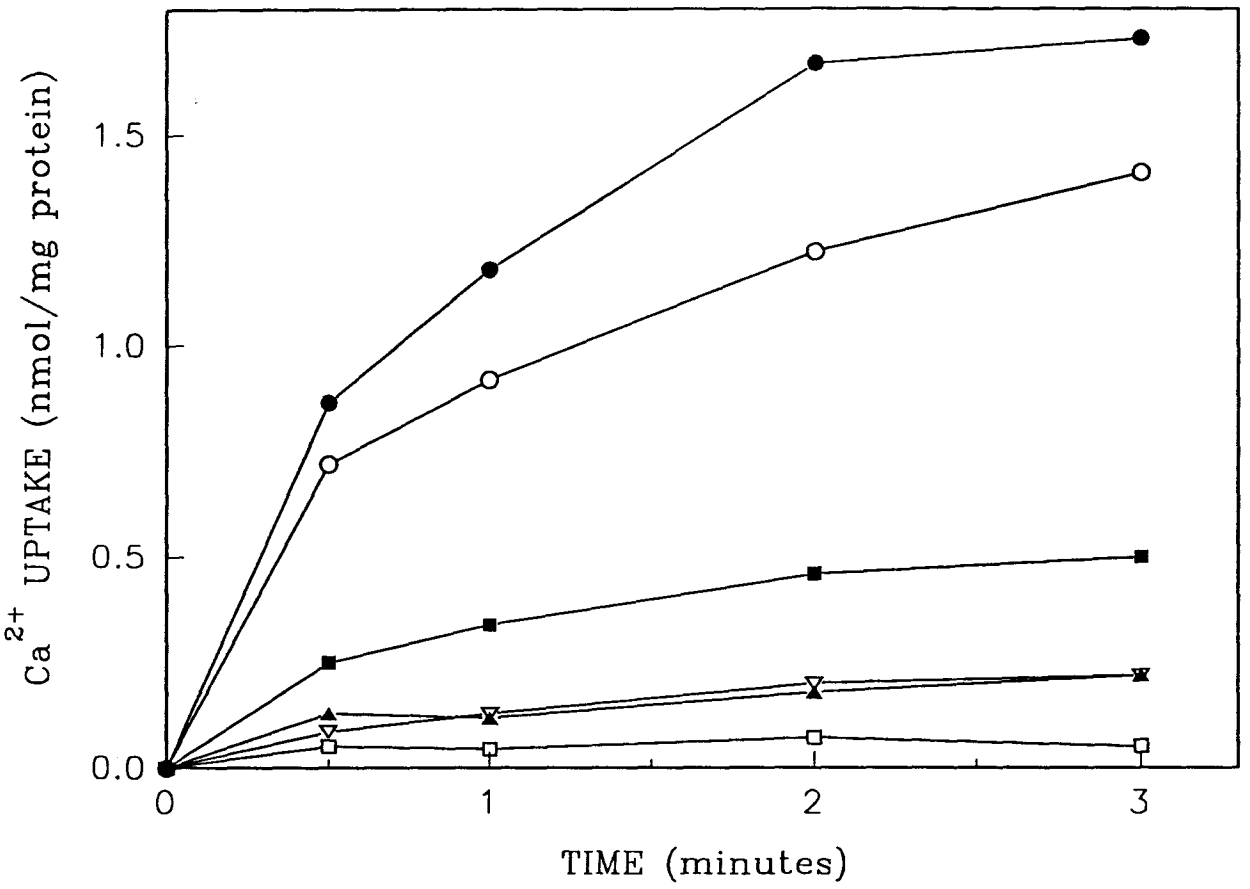

Fig. 11. Inhibition of Ca^{2+} uptake by thapsigargin. Ca^{2+} uptake was determined at 0.5, 1, 2, and 3 min after the addition of ATP in the absence (●) or in the presence of 3 (○), 10 (■), 30 (▽), and 100 nM thapsigargin (▲) under the conditions decribed in Materials and Methods. The action of 5 μM A23187 (□) was also examined. The results represent the mean of 3 separate experiments.

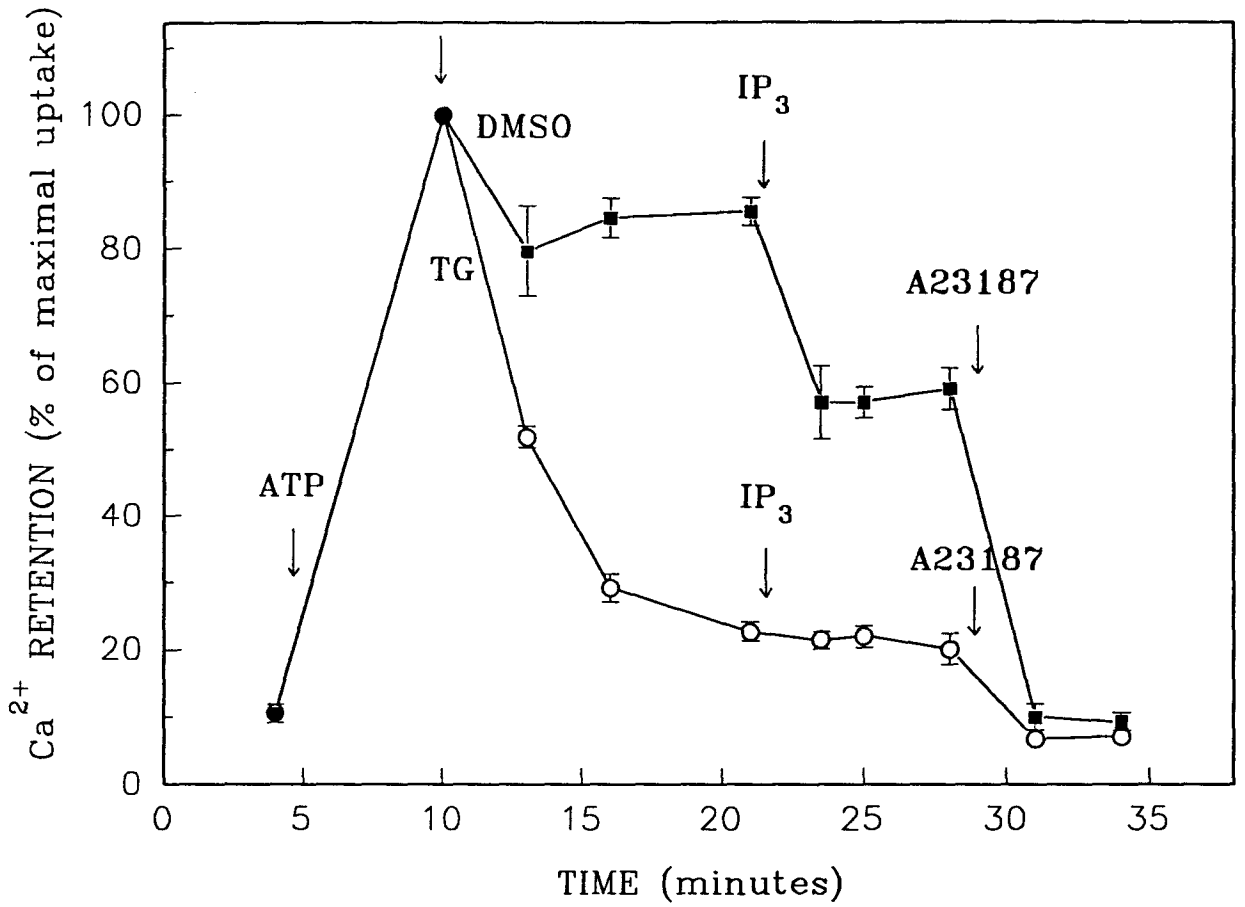

Fig. 12. Depletion of the IP_3-sensitive Ca^{2+} pool by thapsigargin (Tg). PANC-1 microsomes were preloaded with $^{45}Ca^{2+}$ in the presence of ATP. After a maximal uptake of Ca^{2+}, 1 μM Tg (○) or a solvent control, DMSO (■), was added. IP_3 (1 μM) was added 10 min following the additions of Tg and DMSO. A23187 (5 μM) was added near the end of the experiment. Results represent the mean of 3 separate experiments (mean ± S.E.).

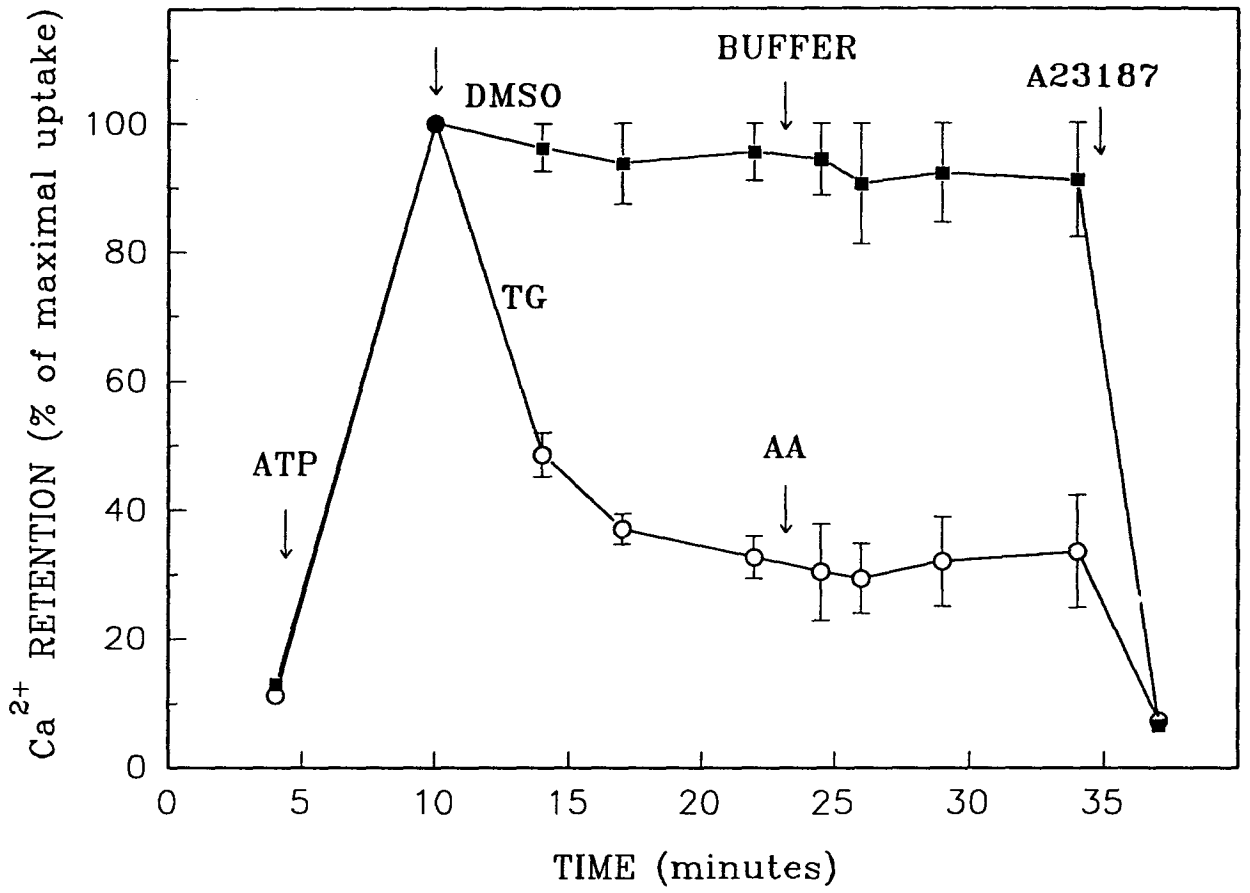

Fig. 13. Depletion of the arachidonic acid (AA)-sensitive Ca^{2+} pool by thapsigargin (Tg). PANC-1 microsomes were preloaded with $^{45}Ca^{2+}$ in the presence of ATP. After a maximal uptake of Ca^{2+}, 1 μM Tg (○) or a solvent control, DMSO (■), was added. AA (100 μM) (○) or a buffer control (■) was added 10 min following the addition of Tg or DMSO. A23187 (5 μM) was added near the end of the experiment. The data shown are the mean of 3 separate experiments (mean ± S.E.).

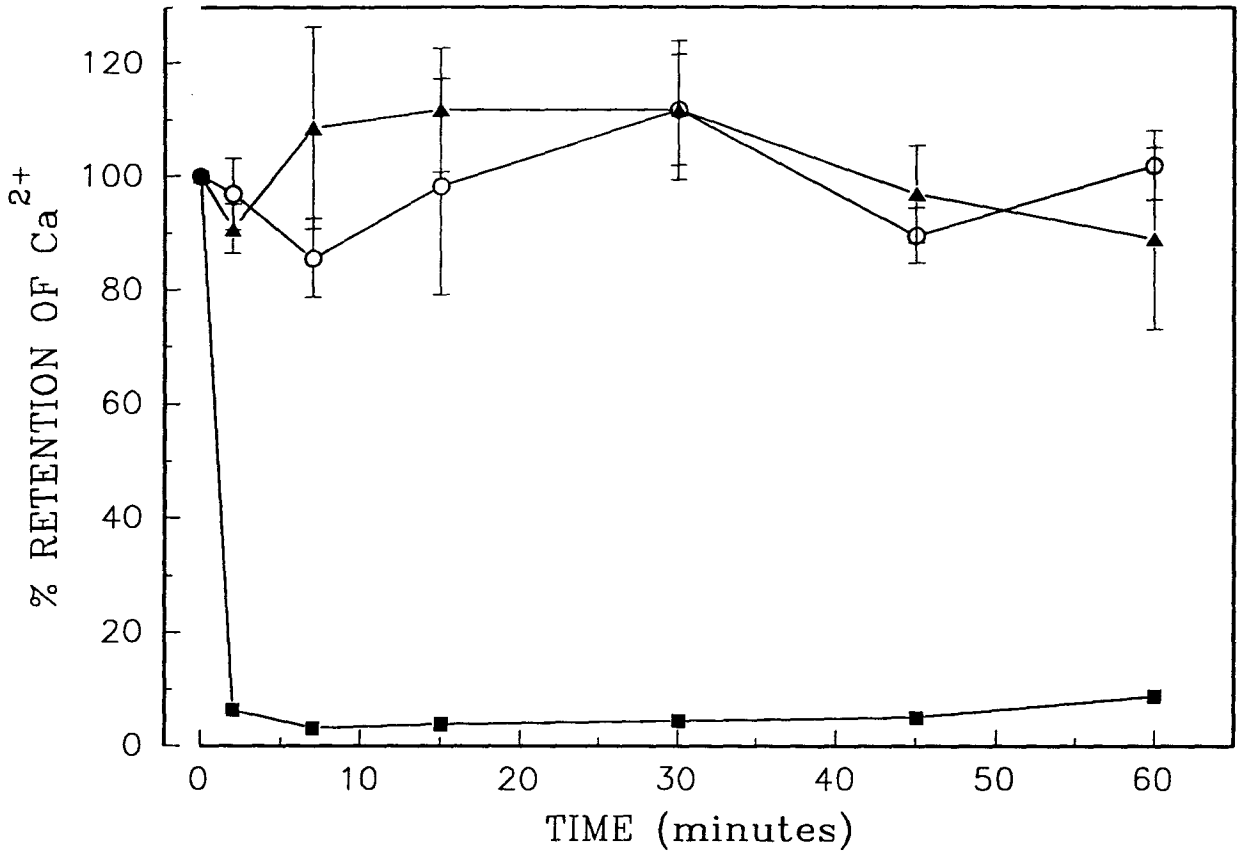

Fig. 14. Arachidonic acid and thapsigargin do not cause Ca^{2+} release from phospholipid vesicles. Phospholipid vesicles (200 nm diameter POPC vesicles; 3 mM lipid) were prepared in buffer containing 1 μM Ca^{2+} and 10 μCi/ml $^{45}Ca^{2+}$. After removal of untrapped calcium, vesicles were incubated at 37° in the presence of 100 μM arachidonic acid (○) or 100 nM thapsigargin (▲) or 1 μM A23187 (■).

that IP_3 and arachidonic acid induced only a subpopulation of the thapsigargin-releasable Ca^{2+} pool.

VI. STUDIES ON POSSIBLE MECHANISM(S) OF ARACHIDONIC ACID- AND THAPSIGARGIN-INDUCED Ca^{2+} RELEASE USING PHOSPHOLIPID VESICLES

The mechanisms by which arachidonic acid and thapsigargin induce Ca^{2+} release from the ER is not known. The possibilities of arachidonic acid and thapsigargin acting as a detergent or as a Ca^{2+} ionophore were investigated using phospholipid vesicles. Liposomes of POPC were loaded with $^{45}Ca^{2+}$ and exposed to arachidonic acid or thapsigargin. Arachidonic acid added at concentration up to 100 μM did not cause the release of entrapped $^{45}Ca^{2+}$, whereas, A23187 induced a massive Ca^{2+} efflux (Fig. 14). Similarly, thapsigargin (100 nM) did not alter the permeability of liposomes to $^{45}Ca^{2+}$ (Fig. 14). These results indicate that arachidonic acid and thapsigargin do not act as a Ca^{2+} ionophore or cause membrane leakiness in lipid vesicles under the conditions specified.

DISCUSSION

The term *Ca^{2+} pool* is widely used in the literature. Apart from the fact that Ca^{2+} pools do exist, little is known about their location, structure, communication and function. One fact which is becoming clear is that Ca^{2+} pools in most cell types are heterogeneous. IP_3 has been shown to release only a fraction of intracellular Ca^{2+} in most studies. Thus, these experiments suggest that there are at least two discrete intracellular Ca^{2+} pools: IP_3-sensitive and IP_3-insensitive. However, other agents, such as GTP, arachidonic acid, and various internal Ca^{2+} pump inhibitors, have been found to release Ca^{2+} from discrete or partially overlapping Ca^{2+} pools independent of the action of IP_3. These studies indicate that there might be multiple functionally distinct Ca^{2+} pools in a given cell type.

In the present studies, we have demonstrated that IP_3, GTP, arachidonic acid and thapsigargin induce the release of accumulated Ca^{2+} from the ER-enriched microsomal membrane fractions of PANC-1 cells, indicating the presence of IP_3-, GTP-, arachidonic acid- and thapsigargin-sensitive Ca^{2+} pools. Our studies also suggest a significant overlap of these Ca^{2+} pools.

I. Ca^{2+} UPTAKE INTO ER-ENRICHED MICROSOMAL MEMBRANE FRACTIONS

PANC-1 microsomal membrane fractions contained a high affinity calcium transporting system, sensitive in the μM free calcium range (Fig. 1), and a similar high affinity calcium activated ATPase activity (Brown and Katz, unpublished data). This Ca^{2+} transporting system driven by an ATP-dependent Ca^{2+} pump was responsible for Ca^{2+} uptake into the ER-enriched microsomes. Values for half-maximal activation of Ca^{2+} uptake into the ER from other cell types have been

reported to vary between 0.1 to 1 μM (Gerok *et al.*, 1990; Ghishan and Arab, 1988; Baquero-Leonis and Pintado, 1989). Our results (K_m = 0.29 ± 0.02 μM) were consistent with most other reports. Initial rates of Ca^{2+} uptake into the ER from other cell types have been shown to be between 6 and 20 nmol/mg/min (Gerok *et al.*, 1990; Ghishan and Arab, 1988). Our value (V_{max} = 15.3 ± 0.3 nmol/mg/min) for PANC-1 cells was also in good agreement with others.

II. IP_3-ACTIVATED Ca^{2+} POOL

A physiological range of IP_3 concentrations in cells is generally reported to be 0.1 (in basal cells) to 1 μM (during stimulation) (Bradford and Rubin, 1986; Tarver *et al.*, 1987; Palmer and Wakelam, 1989). This is a typical response of a second messenger: a relatively small change in its concentration has an enormous impact upon cell function. The effect of IP_3 (range 0.1 to 10 μM) on intracellular Ca^{2+} mobilization was determined in PANC-1 cells. IP_3 at 3 μM caused a maximal Ca^{2+} release from PANC-1 microsomes. IP_3-mediated Ca^{2+} release was found to have a sensitivity (EC_{50} for IP_3 = 0.35 μM) which was consistent with studies by Ghosh *et al.* using microsomes of the DDT_1MF-2 smooth muscle cell line (1988).

In many cell systems, IP_3-degradation rapidly reverses the IP_3-mediated effect (Streb *et al.* 1983, for example). Our studies on IP_3-induced Ca^{2+} release from PANC-1 microsomes were consistent with previous reports. As shown in Fig. 4, IP_3 caused a transient release of the accumulated Ca^{2+}. In some cell systems, however, IP_3 induces a time-sustained release of Ca^{2+} from pre-loaded permeabilized cells (Mullaney *et al.*, 1988, for example).

Our results show that the effect of IP_3 on the release of intracellular Ca^{2+} from microsomal membrane fractions was completely blocked by heparin, an antagonist of the IP_3 receptor. Heparin has been reported to competitively block

the Ca^{2+} -releasing action of IP_3 with a K_i of 3 nM, representing an affinity for the binding site almost 100-fold higher than IP_3 (Ghosh *et al.*, 1988). Presumably, the ring structures of heparin such as glucosamine 2,6-disulfate interact with the IP_3 receptor by mimicking the IP_3 structure (Ghosh *et al.*, 1988).

IP_3-activated Ca^{2+} mobilization can be regulated by cytosolic or extravesicular Ca^{2+}. The present studies in PANC-1 cells demonstrate that extravesicular Ca^{2+} produced an inhibition of IP_3-induced Ca^{2+} release with an IC_{50} of 0.58 μM. Ca^{2+} has been found to be a very potent inhibitor of $[^3H]IP_3$ binding with an IC_{50} of approximately 300 nM (Worley *et al.*, 1987). The ability of Ca^{2+} to inhibit IP_3 binding is mediated through a protein named calmedin (Danoff *et al.*, 1988). A recent study by Mignery *et al.* (1992) however suggests that the regulation of the IP_3 receptor function by Ca^{2+} does not involve its binding site but is apparently due to a regulation of the IP_3-dependent Ca^{2+} channel.

In smooth muscle cells, the effects of cytosolic Ca^{2+} are biphasic: at less than 300 nM $[Ca^{2+}]_i$, Ca^{2+} enhances IP_3 sensitivity, whereas higher $[Ca^{2+}]_i$ are inhibitory (Iino, 1987, 1990). In a study of the rapid kinetics of IP_3 action in synaptosomes, Finch *et al.* (1991) also reported biphasic actions of Ca^{2+}. They showed that both effects of Ca^{2+} occur in the same range (half maximal at about 600 nM), but the inhibitory effect of Ca^{2+} develops more slowly. Both positive and negative feedback regulation may play a role in generating complex Ca^{2+} signals (Finch *et al.*, 1991).

III. GTP-ACTIVATED Ca^{2+} MOVEMENT

In some situations, GTP alone has been reported to cause the direct release of Ca^{2+} from intracellular Ca^{2+} stores independent of IP_3 (Ueda *et al.*, 1986). In others, GTP potentiates IP_3-mediated Ca^{2+} mobilization (Thomas, 1988) by

permitting direct Ca^{2+} movement between IP_3-sensitive and IP_3-insensitive Ca^{2+} pools (Gill *et al.*, 1988). Our results show that the GTP-sensitive pool, which was approximately 3 times larger than the IP_3-sensitive pool, could be released by GTP in the absence of IP_3. The combination of GTP and IP_3 resulted in a greater release than either agent alone. Unlike IP_3, GTP-mediated Ca^{2+} release was completely heparin-insensitive. As heparin directly interacts with and inhibits the IP_3-activated Ca^{2+} channel, the data suggest that GTP-mediated release of Ca^{2+} did not involve the IP_3-activated Ca^{2+} channel.

The mechanism by which GTP induces Ca^{2+} release remains to be determined. However, the action of GTP appeared to be mediated by an enzymatic GTP hydrolytic process since GTPγS had no effect on Ca^{2+} release. The possibility of GTP hydrolytic products, GMP and GDP, being responsible for the GTP-mediated effect was therefore determined (Gill *et al.*, 1986). GMP had no effect on Ca^{2+} release. GDP, however, induced Ca^{2+} release, but only after a lag of 30 to 60 sec probably due to nucleoside diphosphokinase-mediated conversion to GTP. The effect of GDP is blocked by ADP, which competes with GDP for diphosphokinase. In addition, the release could not be mimicked by other nucleotide triphosphates (ATP, ITP and CTP), indicating a relative specificity for GTP (Gill *et al.*, 1986). Recent studies on sea urchin eggs however show that cyclic GMP activates the release of Ca^{2+} from an IP_3-sensitive store via a mechanism independent of the IP_3 receptor (Whalley *et al.*, 1992).

According to Dawson *et al.* (1987), GTP-induced fusion of microsomal vesicles accounted for the GTP-mediation of both Ca^{2+} efflux and potentiation of IP_3-promoted Ca^{2+} release. PEG has often been used to enhance the effect of GTP, probably by promoting vesicle fusion (Dawson *et al.*, 1987). Because fusion of IP_3-sensitive with IP_3-insensitive vesicles would increase the IP_3-sensitive vesicle population, this would lead to potentiation of the IP_3-induced Ca^{2+} release. It can

also be expected that the fusion process itself causes leakage and therefore release of Ca^{2+}. However, in our studies, both PEG and GTP alone had little or no effect on the release of Ca^{2+}. Furthermore, the size of the IP_3-sensitive Ca^{2+} pool was not altered by GTP and PEG (Table II). It has been reported that the action of GTP could be reversed either by washing or by addition of GDP to the GTP-activated permeabilized cells or microsomes (Chueh *et al.*, 1987; Gill *et al.*, 1986). These results argue against a membrane fusion mechanism that would not be expected to be a reversible process. The mechanistic basis for communication between these Ca^{2+} pools is still unknown but appears to be mediated by the low molecular weight G-proteins which regulate membrane traffic within the cell by controlling fusion and other forms of reorganization of intracellular organelles (Bourne, 1988; Bourne *et al.*, 1990; Wilschut, 1989).

Other possible mechanisms have been proposed. Nicchitta and coworkers (1987) found a good correlation between GTP-mediated Ca^{2+} release with a GTP-induced increase in membrane permeability. Studies on hepatic microsomes indicate that inhibition of Ca^{2+}-ATPase appears to play a role, at least partially, in GTP-induced Ca^{2+} release (Kimura *et al.*, 1990).

IV. ARACHIDONIC ACID-MEDIATED Ca^{2+} RELEASE

We have demonstrated effects of arachidonic acid on mobilizing preloaded $^{45}Ca^{2+}$ from microsomes of PANC-1 cells. Other laboratories have shown that arachidonic acid releases intracellular Ca^{2+} from permeabilized pancreatic islets (Wolf *et al.*, 1986), permeabilized human T and B lymphocytes (Corado *et al.*, 1990), human platelet membrane vesicles (Tohmatsu *et al.*, 1989) and rat liver microsomes (Chan and Turk, 1987). The arachidonic acid-mediated effect appeared to be due to a direct action of arachidonic acid and not due to metabolism to

eicosanoids since both indomethacin and NDGA, inhibitors of cyclooxygenase and lipoxygenase, were ineffective in preventing the effect of arachidonic acid. Moreover, as palmitoleic acid is not a substrate for cyclooxygenase or lipoxygenase (Jeffcoat and James, 1984), the Ca^{2+} releasing effect of palmitoleic acid supports our observations that the fatty acid itself, not its metabolite, induces the release of accumulated Ca^{2+} from the microsomes.

The mechanisms involved in the arachidonic acid-mediated effects have been investigated. Arachidonate does not alter microsomal membrane permeability or Ca^{2+}-ATPase activity, but does inhibit Ca^{2+} accumulation (Chan and Turk, 1987). The latter may contribute to the arachidonic acid-induced Ca^{2+} release. Our studies on phospholipid vesicles suggest that arachidonic acid does not alter membrane permeability and therefore does not appear to act as either a Ca^{2+} ionophore or a detergent.

A physiological role for arachidonic acid-mobilizing intracellular Ca^{2+} remains unclear. It has been suggested that arachidonic acid may act as a Ca^{2+} mediator in intact platelets (Tohmatsu *et al.*, 1989), as the agonist-stimulated increase in $[Ca^{2+}]_i$ was reduced in platelets in the presence of PLA_2 inhibitor. This result indicates that arachidonic acid may induce an additional Ca^{2+} release to that of IP_3-mediated release in platelets (Tohmatsu *et al.*, 1989). In pancreatic islet cells, arachidonic acid may participate in glucose-activated Ca^{2+} mobilization and insulin secretion, probably in cooperation with IP_3 (Wolf *et al.*, 1986). These observations indicate that the arachidonic acid-mediated Ca^{2+} mobilizing pathway may co-exist with the IP_3-induced pathway in some cell types. Our studies have demonstrated that at least a significant proportion of the arachidonic acid-releasable and IP_3-sensitive Ca^{2+} pools overlap. However, the action of arachidonic acid (20:4(n-5,8,11,14)) in PANC-1 cells appeared to be nonspecific as other fatty acids [linoleic acid (18:2(n-9,12)), elaidic acid (18:1(n-9)), arachidic acid

(20:0), palmitoleic acid (16:1(n-9)) and stearic acid (18:0)] also mediated the release of Ca^{2+}. In these experiments, polyunsaturated fatty acids were most effective.

V. EFFECT OF THAPSIGARGIN ON INTRACELLULAR Ca^{2+} MOVEMENT

Thapsigargin has been shown to inhibit the Ca^{2+}-ATPase activities of internal Ca^{2+} pumps (Thastrup *et al.*, 1990; Lytton *et al.*, 1991). In these experiments, thapsigargin maximally blocked 85% of the Ca^{2+} uptake into, and induced a rapid release of 85% of preloaded Ca^{2+} from PANC-1 microsomes. The thapsigargin-induced Ca^{2+} release may be directly due to the inhibition of the Ca^{2+}-ATPase activity. Thastrup and coworkers (1990) found thapsigargin induced Ca^{2+} release and inhibition of the Ca^{2+}-ATPase activity with a equivalent dose-dependency. Depletion of ATP which also results in inhibition of Ca^{2+}-ATPase caused a similar Ca^{2+} release (Thastrup *et al.*, 1990). Furthermore, another inhibitor of the intracellular Ca^{2+} pump, BHQ, was also observed to mobilize intracellular Ca^{2+} pools in Fura-2-loaded AR42J cells and pancreatic acini (Muallem *et al.*, 1991).

There are two possible mechanisms by which thapsigargin may induce Ca^{2+} release from PANC-1 microsomes (Fig. 15). As shown in Fig. 2, Ca^{2+} was actively accumulated into the microsomes. Ca^{2+} uptake reached a steady state after 5 min. At the steady state, Ca^{2+} uptake (by active pumping) and release (probably by passive leaking) were in equilibrium. A decrease in Ca^{2+} uptake mediated by thapsigargin would result in net Ca^{2+} release. Our studies on phospholipid vesicles showed that thapsigargin did not alter membrane permeability in a non-specific fashion. A possibility that thapsigargin may act as a Ca^{2+} ionophore was excluded by our studies on phospholipid vesicles and work by others using red blood cells (Foder *et al.*, 1989). Therefore, thapsigargin may induce Ca^{2+} release

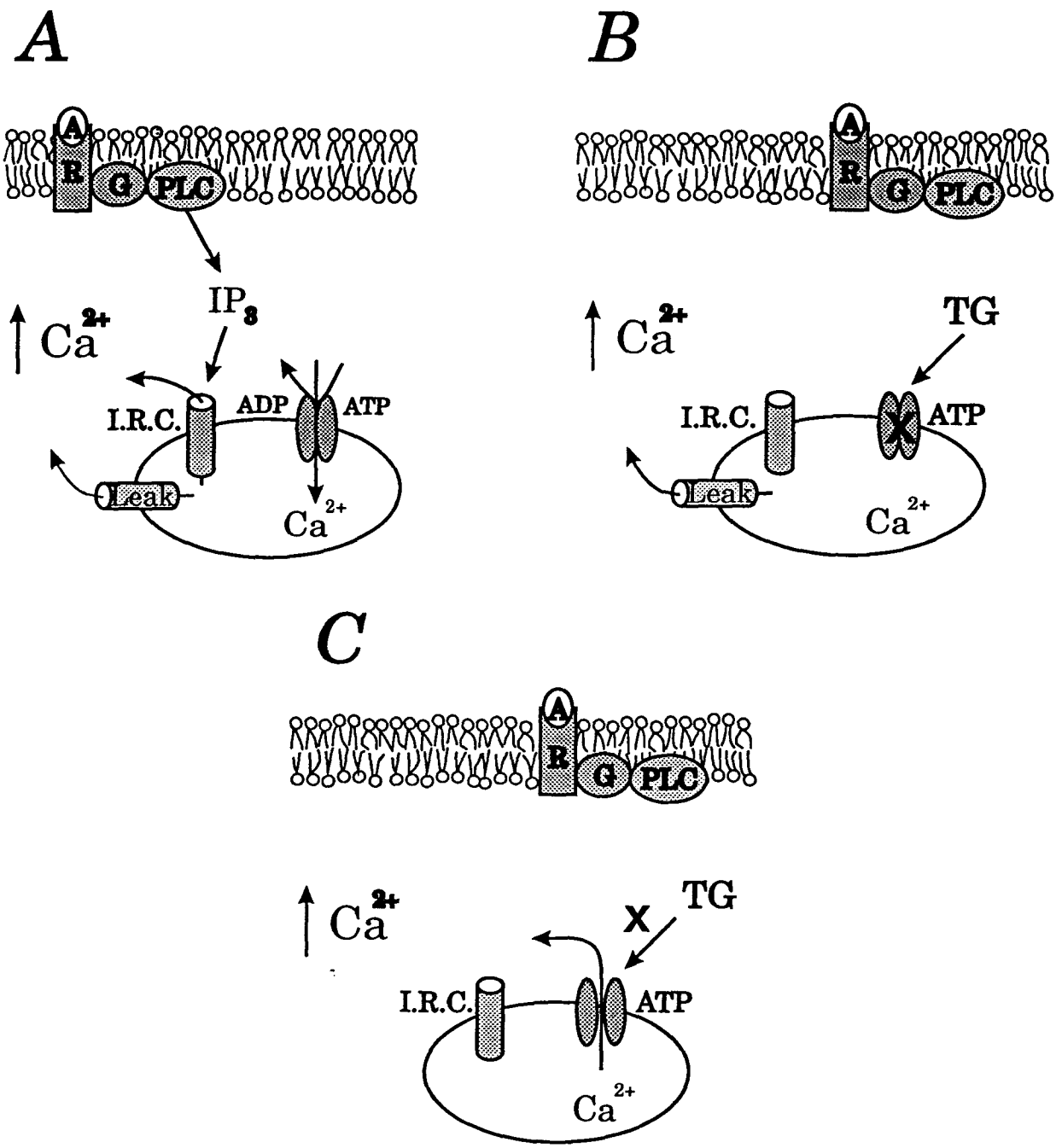

Fig. 15. Possible models for thapsigargin-induced Ca^{2+} release from the ER. ***A***: Agonists (A) activate surface membrane receptors (R), which in turn activate PLC. A guanine nucleotide regulatory protein (G) is involved in coupling receptors to PLC. This leads to the production of IP_3 which induces the release of Ca^{2+} via an IP_3-regulated channel (I.R.C.). Ca^{2+} is sequestered into the ER by a Ca^{2+} pump. ***B***: Thapsigargin (TG) may also release Ca^{2+} from the ER via a mechanism involving inhibition of the Ca^{2+} pump which unmask the activity of a Ca^{2+} leak channel. ***C***: Another possible mechanism by which TG induces Ca^{2+} release is TG may cause the Ca^{2+} pump itself to act as a channel for Ca^{2+} release.

via constitutive Ca^{2+} leaking channels. Another possible mechanism is that thapsigargin may cause the Ca^{2+} pump itself to act as a channel for Ca^{2+} release.

In our studies, it appears that the remaining 15% of the Ca^{2+}-ATPase activity in PANC-1 microsomes was not sensitive to thapsigargin. In contrast, 5 μM A23187 inhibited almost 100% of the Ca^{2+} uptake. A Ca^{2+} pool that is thapsigargin-insensitive but A23187-releasable was also reported by Bian *et al.* (1991) in permeabilized DDT_1MF-2 cells, and by Verma and colleagues (1990) in brain microsomes. However, it appears that there is no functional difference between thapsigargin-sensitive and -insensitive Ca^{2+} pumps (Bian *et al.*, 1991). It is unlikely that the different sensitivity of Ca^{2+}-ATPase to thapsigargin is due to inhibition of different isoforms of the internal Ca^{2+}-ATPase by thapsigargin, since thapsigargin has been shown to inhibit all of the isoforms with equal potency (Lytton *et al.*, 1991).

There is no information at present to indicate the nature or significance of the thapsigargin-insensitive pool. Whether there are unknown components, other than the presently identified Ca^{2+} pumps, involved in the thapsigargin-induced effect remains unclear. Since we know that Ca^{2+}-ATPase of the plasma membrane is not affected by thapsigargin, one possibility is that the thapsigargin-insensitive Ca^{2+} pool could be inside-out plasma membrane vesicles formed during the preparation of microsomes. In addition, Ca^{2+} uptake into nonmitochondrial Ca^{2+} pools of some nonexcitable cells can occur via a H^+-Ca^{2+} exchange mechanism, driven by an ATP-dependent H^+ pump (Thévenod and Schulz, 1988; Thévenod *et al.*, 1989; Schulz *et al.*, 1989). Therefore, another possibility is that the thapsigargin-insensitive pool could be a Ca^{2+} pool which accumulates Ca^{2+} via the H^+-Ca^{2+} exchange.

Interestingly, thapsigargin affects multiple intracellular Ca^{2+} pools. We have demonstrated that thapsigargin depleted the IP_3- or arachidonic acid-

sensitive Ca^{2+} pool. Thastrup *et al.* (1990) reported that the thapsigargin-sensitive Ca^{2+} pool includes both IP_3- and GTP-sensitive pools. Also, thapsigargin has been shown to inhibit Ca^{2+} uptake into IP_3- and GTP-sensitive intracellular stores in permeabilized DDT_1MF-2 smooth muscle cells (Bian *et al.*, 1991). This characteristic of thapsigargin makes it a useful pharmacological tool for studying Ca^{2+} homeostasis.

VI. SIGNIFICANCE OF Ca^{2+} WITHIN THE ER

It appears that the IP_3-induced Ca^{2+} release from the ER is not a whole cellular signalling event. There is growing evidence to suggest that the Ca^{2+} levels within such organelles appear to have important effects on signalling and regulation within cells. As mentioned previously, the emptying of IP_3-sensitive Ca^{2+} stores may be a direct trigger for the activation of Ca^{2+} entry across the plasma membrane (Putney, 1990; Hoth and Penner, 1992). It has also been shown that the level of intraluminal Ca^{2+} may be a key regulator of the IP_3 receptor itself (Irvine, 1990). The luminal Ca^{2+} within the ER is also considered to play a potential role in the control of essential functions of this organelle. The ER is the major site for translation, translocation, and processing of membrane biogenesis and trafficking. Recently, Sambrook (1990) described the possibility that intraluminal ER Ca^{2+} may have a major regulatory influence on these activities. Ghosh *et al.* (1991) reported that thapsigargin-induced depletion of Ca^{2+} within the ER prevents cell division and DNA synthesis, and therefore inhibits cell growth.

VII. GENERAL DISCUSSION

The conclusions of the present study are that Ca^{2+} pools in ER-enriched microsomes of PANC-1 cells are heterogeneous. In addition, we have found that Ca^{2+} can be released by IP_3, GTP, arachidonic acid and thapsigargin by different mechanisms (Fig. 16). Of these Ca^{2+} mediators, the action of IP_3 in the propagation of Ca^{2+} signals is now widely established. However, control of the

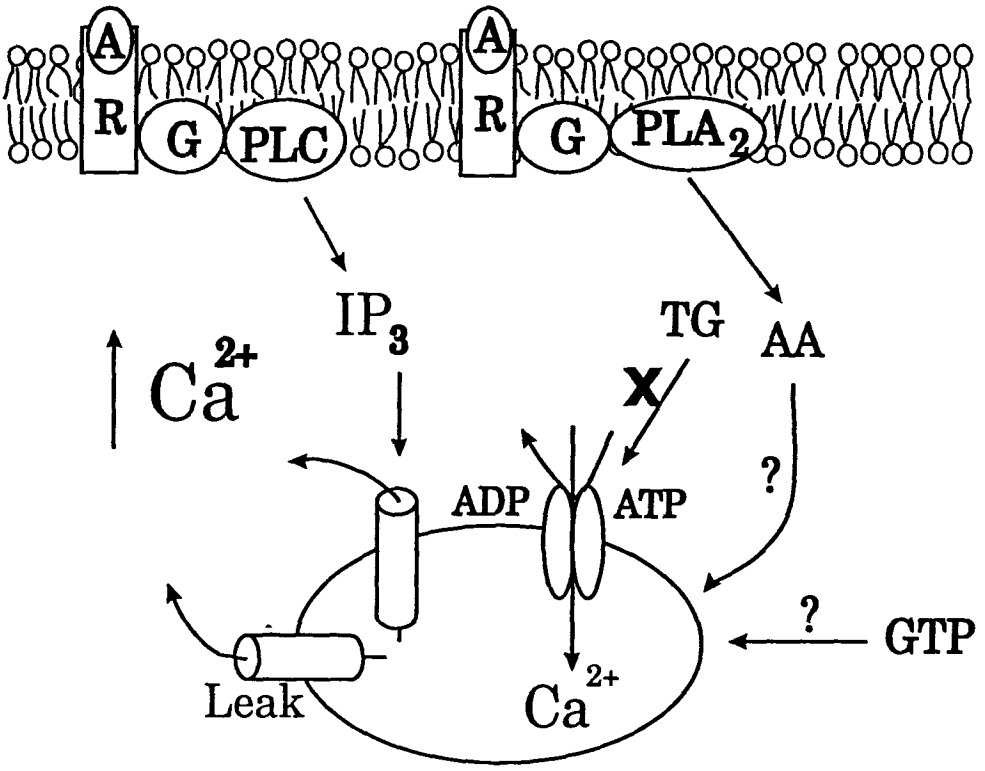

Fig. 16. Pathways for calcium release from the ER-enriched PANC-1 microsomes. Agonists (A) activate surface membrane receptors (R), which in turn activate PLC and/or PLA_2. Guanine nucleotide regulatory proteins (G) are involved in coupling receptors to PLC and PLA_2. The activation of PLC leads to the production of IP_3 which induces the release of Ca^{2+} via an IP_3-regulated channel. Arachidonic acid (AA) generated by PLA_2 pathway also releases Ca^{2+} from the ER via an unknown mechanism. Thapsigargin (TG) mobilizes intracellular Ca^{2+} apparently by inhibition of the ER Ca^{2+} pumps. Ca^{2+} release in response to GTP appears to be mediated by an enzymatic GTP hydrolytic process, however, the precise mechanism(s) of GTP-induced Ca^{2+} release is not clear.

IP_3-sensitive pool and its relationship to other intracellular Ca^{2+} pools remains to be determined. The physiological significance of the GTP- and arachidonic acid-induced intracellular Ca^{2+} movements is still uncertain.

SUMMARY AND CONCLUSIONS

1. The ER-enriched microsomes prepared from PANC-1 cells contained a high affinity calcium transporting system which was responsible for Ca^{2+} accumulation into the ER.
2. Intracellular Ca^{2+} pools in PANC-1 cells are heterogeneous. There are multiple releasable Ca^{2+} pools in this cell type: IP_3-, GTP-, arachidonic acid- and thapsigargin-sensitive pools. The size of these Ca^{2+} pools is variable.
3. IP_3 induced the release of Ca^{2+} from PANC-1 microsomes, which was completely inhibited by heparin, an antagonist of the IP_3 receptor. The IP_3-mediated Ca^{2+} release was also inhibited by extracellular or cytosolic Ca^{2+}. This supports the present view that increased $[Ca^{2+}]_i$ might feed back to further inhibit IP_3-induced Ca^{2+} release.
4. GTP induced the release of the accumulated Ca^{2+} from the microsomal membrane fractions in the absence of IP_3. This effect involves a GTP hydrolytic process, not the IP_3-activated Ca^{2+} channel. GTP did not appear to alter the size of the IP_3-releasable Ca^{2+} pool in our system.
5. Arachidonic acid caûsed a rapid release of the accumulated Ca^{2+}. This effect was not highly specific since other fatty acids induced similar effects. Metabolites of arachidonic acid did not seem to be involved in arachidonic acid-mediated Ca^{2+} release.
6. Thapsigargin induced Ca^{2+} release from PANC-1 microsomes and resulted in a depletion of the IP_3- or arachidonic acid-sensitive Ca^{2+} pool apparently by an inhibition of Ca^{2+} accumulation into the ER.
7. Our studies on phospholipid vesicles suggested that both arachidonic acid and thapsigargin did not exert either a membrane detergent-like or a Ca^{2+} ionophore-like effect.

8. The ER Ca^{2+} pools of PANC-1 cells could be released by various Ca^{2+} mediators via different mechanisms. Significant overlap of these Ca^{2+} pools appear to exist in PANC-1 cells.

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