GTP- and inositol 1,4,5-trisphosphate-induced release of $^{45}\text{Ca}^{2+}$ from a membrane store co-localized with pancreatic-islet-cell plasma membrane

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Inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], arising from hydrolysis of phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$], is proposed as the link between membrane-receptor activation and mobilization of Ca^{2+} from intracellular sites in hormone-secreting cells. The location of $\text{Ins}(1,4,5)\text{P}_3$ -sensitive membranes was investigated in cultured neonatal β -cells. Membranes were obtained after lysis of cells attached to positively charged Sephadex. After lysis the presence of the enzyme markers 5'-nucleotidase, glucose-6-phosphatase, NADH-cytochrome *c* reductase, UDP-galactosyltransferase and succinate dehydrogenase indicated the mixed nature of the preparation. After sonication, however, UDP-galactosyltransferase and succinate dehydrogenase activities were undetectable, but 4.8 % of total cellular glucose-6-phosphatase and 3.4 % of total cellular NADH-cytochrome *c* reductase remained with 5'-nucleotidase in the preparation, indicating endoplasmic-reticulum association. ATP-dependent $^{45}\text{Ca}^{2+}$ accumulation was shown in this preparation (410 ± 24 pmol/mg of protein at 150 nM free Ca^{2+}) and was inhibited by vanadate (100 μM). Ca^{2+} release was effected by $\text{Ins}(1,4,5)\text{P}_3$, with half-maximal release at 0.5 ± 0.14 μM - $\text{Ins}(1,4,5)\text{P}_3$, $t_{1/2}$ 11.2 ± 1.1 s. GTP- and guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG)-promoted release of $^{45}\text{Ca}^{2+}$ was demonstrated in this preparation, but the kinetics of release (half-maximal Ca^{2+} release at 5.4 ± 0.7 μM , with $t_{1/2}$ 77.3 ± 6.9 s, and at 51.1 ± 4.2 μM , with $t_{1/2}$ 19.0 ± 2.2 s, for GTP and p[NH]ppG respectively), and the ability of neomycin sulphate to block p[NH]ppG-induced release only, are indicative of separate release mechanisms after treatment with these agents. A close association between plasma membrane and elements of the endoplasmic reticulum is indicated in this model, providing a possible mechanism for local alterations in free Ca^{2+} in the sub-plasma-membrane region.

INTRODUCTION

Intracellular free Ca^{2+} concentration is widely recognized to influence stimulus-coupled responses in numerous eukaryotic cells, including the pancreatic β -cell. A mechanism for the close relationship exhibited between phosphoinositide turnover and Ca^{2+} mobilization activated by receptor-mediated mechanisms has been elucidated by the demonstration that inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], the water-soluble product of phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$], releases sequestered Ca^{2+} from permeabilized cells (Streb *et al.*, 1984a; Biden *et al.*, 1984; Brass & Joseph, 1985; Taylor & Putney, 1985; Wolf *et al.*, 1985; Ueda *et al.*, 1986) and microsomal fractions (Burgess *et al.*, 1984; Prentki *et al.*, 1984a; Joseph *et al.*, 1984; Wolf *et al.*, 1985; Volpe *et al.*, 1985; O'Rourke *et al.*, 1985; Dawson, 1985; Gill *et al.*, 1986).

The distribution of enzyme markers indicated that $\text{Ins}(1,4,5)\text{P}_3$ -sensitive microsomal vesicles are derived from the endoplasmic reticulum, where saturable receptors for $\text{Ins}(1,4,5)\text{P}_3$ are located (Baukal *et al.*, 1985; Spat *et al.*, 1986) and in which a (Mg^{2+} + ATP)-dependent Ca^{2+} -transporting activity associated with regulation of cytosolic Ca^{2+} has been demonstrated (Prentki *et al.*, 1984b). $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release has not been

shown from purified plasma membranes (Streb *et al.*, 1984b).

However, the demonstration of a plasma-membrane location for the $\text{Ins}(1,4,5)\text{P}_3$ phosphatase responsible for $\text{Ins}(1,4,5)\text{P}_3$ degradation (Storey *et al.*, 1984; Seyfred *et al.*, 1984; Rana *et al.*, 1986) would indicate a functional proximity between $\text{Ins}(1,4,5)\text{P}_3$ release, action and inactivation. A spatial precedent for this is found in electron-microscopic studies which describe the similarity of the junction between plasma membranes and the endoplasmic reticulum in cerebellar Purkinje cells and the T-system membranes in sarcoplasmic-reticular/muscle-cell plasma-membrane junctions (Henkart *et al.*, 1976). In living muscle cells an enhanced Ca^{2+} concentration of this spatial component has been confirmed (Williams *et al.*, 1985). In fibroblastic L-cells the endoplasmic reticulum forms morphologically specialized appositions with the plasma membrane, from which Ca^{2+} release is proposed after surface activation (Henkart & Nelson, 1979). In *Limulus* photoreceptors, prominent cisternae of the smooth endoplasmic reticulum, uniquely localized to the R-lobe and immediately underlying the plasma membrane, are sites of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release (Payne & Fein, 1987). Experimental observations of intracellular Ca^{2+} in rat hepatocytes have led Poggioli *et al.* (1985) to propose a plasma-membrane binding site from which receptor action might cause the release of

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate.

Ca^{2+} , subsequently activating the Ca^{2+} -entry pathway. As an extension of this, Putney (1986) has presented a capacitative model in which plasma-membrane Ca^{2+} -entry sites can be regulated by $\text{Ins}(1,4,5)\text{P}_3$ -controlled Ca^{2+} release from the endoplasmic reticulum. In addition to $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from endoplasmic reticulum, GTP-induced Ca^{2+} release has also been described (Dawson, 1985; Gill *et al.*, 1986; Ueda *et al.*, 1986; Dawson *et al.*, 1986; Henne & Söling, 1986; Wolf *et al.*, 1987; Henne *et al.*, 1987). Several observations of the Ca^{2+} -dependency of this process suggest that the guanine-nucleotide-regulated site is distinct from that interactive with $\text{Ins}(1,4,5)\text{P}_3$ (Henne & Söling, 1986). More recently, subcellular-fractionation studies have presented evidence for a rough-endoplasmic-reticulum location for 5'-GTP-releasable Ca^{2+} in guinea-pig parotid acini (Henne *et al.*, 1987). Those authors conclude, however, that $\text{Ins}(1,4,5)\text{P}_3$ -sensitive endoplasmic reticulum is of an identical density with, or is tightly associated with, the plasma membrane.

In a preparation of islet-cell membranes obtained after attachment of cultured cells to positively charged Sephadex, hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ has been shown in response to muscarinic agonists and p[NH]ppG , but not to GTP (Dunlop & Larkins, 1986). We now report that in this preparation, shown to contain enzyme markers for plasma membrane and endoplasmic reticulum, $\text{Ins}(1,4,5)\text{P}_3$ binding is present and $\text{Ins}(1,4,5)\text{P}_3$ -induced $^{45}\text{Ca}^{2+}$ release can be shown under phosphorylating conditions, which support a close functional apposition between these two membrane elements.

MATERIALS AND METHODS

Preparation of islet-cell membranes

Cells from pancreatic islets of neonatal rats, less than 24 h old, were grown in monolayer culture in medium RPMI 1640 for 3 days and attached to hydrated Cytodex 1 beads as described by Dunlop & Larkins (1984a).

β -Cells were washed in Hanks balanced salt solution containing 10 mg of EDTA/ml. Cells were detached by incubation for 4 min at 37 °C with 2 ml of Hanks balanced salt solution, pH 7.4, containing 5 mg of trypsin and 20 mg of EDTA. Single-cell suspension was incubated together with an excess of positively charged Sephadex beads (Cytodex 1).

After attachment at pH 5.2 in 46.5 mM-acetate/217 mM-sucrose buffer, cells were lysed in 10 mM-Tris, pH 8.0. After 15 min cells were washed and samples taken from enzyme determinations. A separate sample of washed membranes was sonicated for 5 s at 50 W in a medium containing 20 mM-NaCl, 100 mM-KCl, 50 mM- MgSO_4 , 25 mM- NaHCO_3 and 0.96 mM- NaH_2PO_4 , pH 7.27 at 22 °C. After sonication, beads and attached membranes were washed twice in this medium.

Membrane characterization

Beads with attached membranes were taken for the determination of 5'-nucleotidase (Dunlop & Larkins, 1984a), glucose-6-phosphatase (Swanson, 1950), NADH-cytochrome *c* reductase (Dallner *et al.*, 1966), UDP-galactosyltransferase (Bretz & Staubli, 1972), succinate dehydrogenase (Pennington, 1961), DNA and immunoreactive insulin (Dunlop & Larkins, 1984a). Additionally, beads with attached membranes from cells

which had been preincubated with fluorescein isothiocyanate-Dextran 70000 were examined for fluorescence remaining in endocytotic vesicles (Galloway, 1983).

ATP-dependent Ca^{2+} uptake and release

Ca^{2+} uptake by attached membranes was measured in the presence and the absence of 0.2 mM-ATP, in a medium containing 20 mM-NaCl, 100 mM-KCl, 5.0 mM- MgSO_4 , 25 mM- NaHCO_3 , 0.96 mM- NaH_2PO_4 and Ca^{2+} /EGTA buffered to give a final calculated free $[\text{Ca}^{2+}]$ of 150 nM at pH 7.5 (Perrin & Sayce, 1967), using the following logarithms of association constants: $\text{Ca}\cdot\text{EGTA}^{4-}$, 10.7160; $\text{Ca}\cdot\text{HEGTA}^{3-}$, 5.330; $\text{Mg}\cdot\text{EGTA}^{4-}$, 5.210; $\text{Mg}\cdot\text{HEGTA}^{3-}$, 3.370; $\text{H}^+\cdot\text{EGTA}^{4-}$, 9.460; $\text{H}^+\cdot\text{HEGTA}^{3-}$, 8.850; $\text{H}^+\cdot\text{H}_2\text{EGTA}^{2-}$, 2.680; $\text{H}^+\cdot\text{H}_3\text{EGTA}^-$, 2.000; $\text{Ca}\cdot\text{ATP}^{4-}$, 3.982; $\text{Ca}\cdot\text{HATP}^{3-}$, 1.800; $\text{Mg}\cdot\text{ATP}^{4-}$, 4.324; $\text{Mg}\cdot\text{HATP}^{3-}$, 2.740; $\text{H}^+\cdot\text{ATP}^{4-}$, 6.950; $\text{H}^+\cdot\text{HATP}^{3-}$, 4.050 (Bjerrum *et al.*, 1957). Incubations were in a final volume of 500 μl containing 15 μg of membrane protein and ^{45}Ca (1 $\mu\text{Ci/ml}$).

Incubations were terminated by rapid (1 s) centrifugation at 15850 *g*. Beads and attached membranes were washed twice in the ionic medium without added ATP, and the ^{45}Ca content of beads and membranes was determined by liquid-scintillation counting of a gel made with water/pseudocumene scintillation cocktail (2:5, v/v). When present, oxalate and vanadate were added during the uptake period. Rapid additions of $\text{Ins}(1,4,5)\text{P}_3$, GTP, p[NH]ppG or ionophore A23187, alone or together with neomycin sulphate, were made at 5 min, and incubation was continued for a further 5–300 s before termination as above.

Binding of *myo*-inositol 1,4,5-[5- ^{32}P]trisphosphate ($\text{Ins}[5\text{-}^{32}\text{P}]\text{P}_3$)

$\text{Ins}[5\text{-}^{32}\text{P}]\text{P}_3$ (approx. 1000 Ci/mmol) was incubated with the lysed and sonicated membrane preparation in the above medium containing ATP and with the addition of 1 mM-2,3-bisphosphoglycerate (Baukal *et al.*, 1985). Displacement of bound $\text{Ins}(1,4,5)[^{32}\text{P}]\text{P}_3$ was determined after $\text{Ins}(1,4,5)\text{P}_3$ addition, with non-specific binding determined in the presence of 10 μM - $\text{Ins}(1,4,5)\text{P}_3$. Assays were incubated for 5 min at 5 °C and were terminated by centrifugation, with rapid washing. The binding to Cytodex beads only was subtracted to obtain specific binding, and the kinetics of binding were determined by Scatchard analysis.

Materials

$\text{Ins}(1,4,5)[^{32}\text{P}]\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and ^{45}Ca were obtained from Amersham International; Cytodex 1 was from Pharmacia Fine Chemicals; GTP and p[NH]ppG were from Boehringer Mannheim; and all other chemicals were from Sigma Chemical Co.

RESULTS

In these preparations, attachment of plasma membrane to beads was shown by a predominance of 5'-nucleotidase enzyme activity. However, in addition the intracellular enzyme markers glucose-6-phosphatase, NADP-cytochrome *c* reductase, UDP-galactosyltransferase and succinate dehydrogenase indicated the additional presence of internal membranes for endoplasmic reticulum, Golgi and mitochondria respectively in this

Table 1. Subcellular enzyme markers in membranes attached to positively charged Sephadex (Cytodex 1)

Islet-cell membranes attached to Cytodex 1 were prepared as described in the Materials and methods section. The values shown are means $\pm$ S.E.M. for three individual preparations of lysed or lysed and sonicated membranes, from which blank determinations (Cytodex 1 beads only) have been subtracted, and are expressed as $\mu\text{mol/h}$ per mg of protein.

Marker	Membrane preparation	
	Lysed	Lysed, sonicated
5'-Nucleotidase	4.47 ± 0.24	5.92 ± 0.71
Glucose-6-phosphatase	1.36 ± 0.19	0.09 ± 0.02
NADH-cytochrome <i>c</i> reductase	1.71 ± 0.16	0.05 ± 0.01
UDP-galactosyltransferase	0.24 ± 0.04	Undetected
Succinate dehydrogenase	0.21 ± 0.02	Undetected

lysed preparation (Table 1). After sonication of this preparation, the preparation remained enriched for plasma-membrane marker enzyme, but contained 4.8% of total cellular glucose-6-phosphatase activity and 3.4% of NADH-cytochrome *c* reductase, although Golgi and mitochondrial-membrane enzymes were below detection limits. No DNA or immunoreactive insulin was detected in either preparation, neither was fluorescein isothiocyanate-dextran fluorescence detected. At 150 nM free Ca^{2+} , ATP-dependent $^{45}\text{Ca}^{2+}$ uptake was demonstrated in these lysed and lysed and sonicated preparations: 1460 ± 140 and 410 ± 24 pmol/mg of protein respectively. Because after lysis only right-side-out vesicles and unsealed elements of the plasma membrane may be expected, further investigations were made only

Fig. 1. Time course of Ca^{2+} uptake in lysed and sonicated membranes attached to positively charged Sephadex (Cytodex 1)

Ca^{2+} uptake by attached membranes was measured in the absence and the presence of ATP. Incubations were terminated by rapid (1 s) centrifugation. Beads and attached membranes were washed, and the ^{45}Ca content of beads and membranes was determined by liquid-scintillation counting and corrected for non-specific binding of ^{45}Ca to beads alone. The results are presented as means $\pm$ S.E.M. (six determinations) in the presence (○) or absence (□) of ATP.

Fig. 2. Effect of inclusion of $\text{Ins}(1,4,5)\text{P}_3$, $\text{p}[\text{NH}]\text{ppG}$ or GTP on $^{45}\text{Ca}^{2+}$ content of lysed and sonicated membranes attached to Cytodex 1

Islet-cell membranes attached to Cytodex 1 were prepared as described in the Materials and methods section. The dose-dependent release of $^{45}\text{Ca}^{2+}$ from the preparation in the presence of $\text{Ins}(1,4,5)\text{P}_3$ (a), $\text{p}[\text{NH}]\text{ppG}$ (b) and GTP (c) was determined over a stimulation period of 10 s for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{p}[\text{NH}]\text{ppG}$ and 25 s for GTP. The time courses of ^{45}Ca release for $\text{Ins}(1,4,5)\text{P}_3$, $\text{p}[\text{NH}]\text{ppG}$ and GTP at 0.5 μM , 50 μM and 5 μM respectively are indicated in the corresponding insets. The results are means $\pm$ S.E.M. for quadruplicate determinations from multiple membrane preparations.

Table 2. ATP-dependent Ca^{2+} uptake in membranes attached to Cytodex 1 after lysis and sonication

ATP-dependent Ca^{2+} uptake was determined in the presence of 150 nM free Ca^{2+} over 5 min as described in the Materials and methods section. The values are means $\pm$ S.E.M. for four to six determinations of the Ca^{2+} content of the membrane (pmol/mg of protein).

Addition	Ca^{2+} content (pmol/mg of protein)
During uptake	
None	410 $\pm$ 24
Oxalate (10 mM)	560 $\pm$ 42
Vanadate (0.15 mM)	208 $\pm$ 19
After uptake	
None	410 $\pm$ 24
Ins(1,4,5) P_3 (10 μM)	217 $\pm$ 27
GTP (10 μM)	189 $\pm$ 21
p[NH]ppG (0.1 mM)	276 $\pm$ 20
A23187 (2 μM)	74 $\pm$ 6.8
Ins(1,4,5) P_3 , neomycin sulphate (1 mM)	294 $\pm$ 32
GTP, neomycin sulphate (1 mM)	192 $\pm$ 14
p[NH]ppG, neomycin sulphate (1 mM)	396 $\pm$ 26

in the preparations which had been both lysed and sonicated.

The time course and ATP-dependence of $^{45}\text{Ca}^{2+}$ uptake into the vesiculated preparation is shown in Fig. 1. In the presence of ATP, addition of the ionophore A23187 (2 μM) decreased the Ca^{2+} content of the preparation to 74 $\pm$ 6.8 pmol/mg of protein. When incubated in the presence of vanadate over the concentration range 0–3 mM, inhibition of $^{45}\text{Ca}^{2+}$ uptake was seen (threshold 100 μM -vanadate, half-maximal inhibitory concentration 150 μM). The non-linear Ca^{2+} uptake shown in Fig. 1 was enhanced and became linear in the presence of oxalate (10 mM) at 150 nM free Ca^{2+} (calculated by using the logarithm of association constant for $\text{Ca} \cdot \text{oxalate}$, 2,300, in the calculation of free Ca^{2+} described in the Materials and methods section).

Further characterization was done in the absence of oxalate. Addition of Ins(1,4,5) P_3 , p[NH]ppG and GTP induced the rapid release of Ca^{2+} from the preparation. The concentration-dependence and time course for these agents is shown in Fig. 2. Half-maximal Ca^{2+} release occurred with 0.5 $\pm$ 0.14 μM -Ins(1,4,5) P_3 , with maximal release at 5 μM -Ins(1,4,5) P_3 ; the $t_{1/2}$ was calculated as 11.2 $\pm$ 1.1 s. GTP and p[NH]ppG elicited Ca^{2+} release; half-maximal release occurred at 5.4 $\pm$ 0.7 μM and 51.1 $\pm$ 4.2 μM respectively, and the $t_{1/2}$ for Ca^{2+} release was calculated as 77.3 $\pm$ 6.9 and 19.0 $\pm$ 2.2 s respectively. The inclusion of neomycin sulphate (1 mM) inhibited Ca^{2+} release induced by p[NH]ppG, but not by GTP (Table 2).

Ins(1,4,5)[^{32}P] P_3 binding could be demonstrated in this preparation, half-maximal decrease in specific binding occurring at 3.4 $\pm$ 0.4 nM-Ins(1,4,5) P_3 (five determinations), and Scatchard analysis indicated values for K_D and B_{max} of 3 nM and 17.2 fmol/mg of protein respectively.

DISCUSSION

The preparative technique relies on the binding of plasma membranes at pH 5.2, with subsequent hypo-

osmotic lysis and washings at pH 8.0, which is below the pH optimum for re-attachment of intracellular membranes. Binding of an endoplasmic-reticulum-enriched fraction obtained by sucrose-gradient centrifugation was shown not to occur to the beads or to the beads and attached membranes at the lysis pH. It is therefore proposed that the endoplasmic-reticulum elements present in the sonicated preparation have remained associated, through lysis and sonication, with the inner surface of the attached plasma membrane; transmission electron microscopy has confirmed the presence of heterogeneous vesicles (0.17 $\pm$ 0.04 μm diameter, $n = 100$) associated with the attachment face of membrane to the bead (M. E. Dunlop & R. G. Larkins, unpublished work).

The findings shown in Table 2 of oxalate-supported vanadate-inhibitable Ca^{2+} uptake are a strong indication of the presence of vesiculated endoplasmic reticulum in the preparation.

We were not able to demonstrate endocytosis-derived endosomal vesicles, nor could plasma-membrane vesicles be expected. Earlier documentation of plasma membrane prepared by adhesion of cells to polylysine-coated glass beads showed that sonication removes all plasma membrane not apposed to the bead surface, leaving a membrane of known orientation without plasma-membrane vesiculation (Kalish *et al.*, 1978). This is supported in the present study by the availability of Ins(1,4,5) P_3 -binding sites with K_D values similar to those reported previously, although the considerable decrease in abundance as compared with previous determinations in permeabilized cells or microsomal preparations (Baukal *et al.*, 1985; Spat *et al.*, 1986, 1987) indicates that only part of the Ins(1,4,5) P_3 -sensitive endoplasmic reticulum is sampled in this preparation.

The findings for Ins(1,4,5) P_3 -induced Ca^{2+} release are in agreement with previous findings in a number of permeabilized cell preparations and preparations enriched in endoplasmic reticulum (Burgess *et al.*, 1984; Brass & Joseph, 1985; Wolf *et al.*, 1985; O'Rourke *et al.*, 1985; Ueda *et al.*, 1986).

Similarly, the release of Ca^{2+} by GTP agrees with findings in permeabilized cells and microsomes of N1E-115 murine neuroblastoma (Ueda *et al.*, 1986) and rat islets (Wolf *et al.*, 1987). However, in contrast, GTP-mediated Ca^{2+} release in the present study was effected without addition of the poly(ethylene glycol), which was required in N1E-115 microsomal preparations (Ueda *et al.*, 1986; Gill *et al.*, 1986), and was shown to be a requirement for Ca^{2+} release mediated by the combined stimulus of GTP and Ins(1,4,5) P_3 in liver microsomes (Dawson, 1985). It was speculated that homogenization during sample preparation may have dissociated or damaged endoplasmic-reticulum components. Dawson *et al.* (1987) have shown that GTP-induced Ca^{2+} release from poly(ethylene glycol)-treated liver microsomal vesicles requires their association into large, often multilayered, vesicles. More recently it has been shown that close membrane association rather than membrane fusion suffices for GTP-induced Ca^{2+} release in neuronal and smooth-muscle cells (Chueh *et al.*, 1987). It is suggested from the present study that such elements remain functionally intact.

Ca^{2+} release mediated by p[NH]ppG is an apparently disparate finding; this non-hydrolysable analogue of GTP cannot be shown to elicit Ca^{2+} release in microsomal

preparations studied previously. The present finding may be due to p[NH]ppG -induced activation of $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase, which has been shown in these membranes (Dunlop & Malaisse, 1986; Dunlop & Larkins, 1986), the resulting $\text{Ins}(1,4,5)\text{P}_3$ being effective in releasing Ca^{2+} . This is supported by the finding that inclusion of neomycin sulphate inhibited p[NH]ppG -induced, but not GTP-induced, Ca^{2+} release.

The selective binding of polycationic antibiotics to $\text{PtdIns}(4,5)\text{P}_2$, thereby preventing phosphodiesteratic cleavage (Schibeci & Schacht, 1977; Marche *et al.*, 1983), or the recently described direct $\text{Ins}(1,4,5)\text{P}_3$ -concentration-dependent interaction of neomycin with $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} release (Prentki *et al.*, 1986), may be operative. In the present study neomycin sulphate inhibits $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release to a small but non-significant extent, supporting the postulate that its major mechanism of action in inhibiting p[NH]ppG -mediated Ca^{2+} release is via inhibition of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis. In contrast with p[NH]ppG , GTP-mediated Ca^{2+} release was not inhibited in the presence of neomycin sulphate. This finding, together with the differences seen in magnitude of the available pool, dose response and time course, strongly suggest a separate release mechanism utilized by GTP distinct from $\text{Ins}(1,4,5)\text{P}_3$ or p[NH]ppG . The mechanism for both remains unknown. From this model, areas of close association between plasma membrane and endoplasmic reticulum may be proposed, providing a possible mechanism for local alterations in free Ca^{2+} in the sub-plasma-membrane region.

Pancreatic insulin secretion requires initial mobilization of intracellular Ca^{2+} , independent of extracellular Ca^{2+} , and is subsequently sustained by extracellular Ca^{2+} entry, which is sensitive to plasma-membrane Ca^{2+} -channel antagonists (Prentki & Wollheim, 1984). We attach considerable functional importance to a demonstration of an $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store in close proximity to the plasma membrane, which indicates that local fluctuations in Ca^{2+} concentrations are possible after hydrolysis of plasma-membrane $\text{PtdIns}(4,5)\text{P}_2$.

Rapid agonist-induced turnover of $\text{PtdIns}(4,5)\text{P}_2$ has been shown in the pancreatic islet (Clements & Rhoten, 1976; Laychock, 1983; Dunlop & Larkins, 1984b; Best & Malaisse, 1984; Wollheim & Biden, 1986). The preparation in the present study describes the elements of a capacitative model for further investigation in these secretory cells.

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