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The digitonin-permeabilized pancreatic islet model

Effect of *myo*-inositol 1,4,5-trisphosphate on Ca^{2+} mobilization

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Glucose-induced insulin secretion is thought to be mediated by submicromolar increases in intracellular Ca^{2+}, although the intracellular processes are not well understood. We have used the previously characterized digitonin-permeabilized insulin-secreting pancreatic islet model to study the role of *myo*-inositol 1,4,5-trisphosphate (IP_3), a putative second messenger for mobilization of intracellular Ca^{2+}. Ca^{2+} efflux from the endoplasmic reticulum was studied with or without vanadate present to inhibit Ca^{2+} reuptake. IP_3 (10 μM), at a free Ca^{2+} level of 0.06 μM, increased Ca^{2+} release by 30% and, when vanadate was present, by 50%. Maximal and half-maximal Ca^{2+} release was observed at 10 μM- and 2.5 μM-IP_3, respectively. IP_3 provoked a rapid release that was followed by slow reuptake. Reuptake was diminished in the presence of vanadate. Inositol 1,4-bisphosphate, inositol 1-phosphate and other phosphoinositide metabolites did not have any significant effect. Because increases in Ca^{2+} levels in the submicromolar range have been previously shown to induce insulin release in digitonin-permeabilized islets, our results are consistent with the concept of IP_3 serving as a second messenger for insulin secretion.

Glucose is widely accepted as being the major stimulus for insulin secretion by the pancreatic β cell (Hedeskov, 1980). However, the intracellular links between initiation and the final step of glucose-induced insulin secretion are not well known (McDaniel *et al.*, 1984). Intracellular Ca^{2+} is thought to be involved in the cellular control of insulin secretion (Wolheim & Sharp, 1981; McDaniel *et al.*, 1984). Recent studies have suggested that the endoplasmic reticulum has an important role in the regulation of intracellular Ca^{2+} (Colca *et al.*, 1983*a*, 1984). Furthermore, we have previously shown in the digitonin-permeabilized islet that increases of Ca^{2+} concentrations in the submicromolar range induce insulin secretion, possibility by activation of a particulate Ca^{2+}- and calmodulin-dependent protein kinase (Colca *et al.*, 1985).

It has been recently proposed that IP_3, the product of agonist-induced phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate, could act as a second messenger for a wide variety of neurotransmitters and peptides by mobilization of intracellular Ca^{2+} (for reviews see Berridge, 1984, and Berridge & Irvine, 1984; see also Berridge *et al.*, 1984; Biden *et al.*, 1984; Burgess *et al.*, 1984; Gershengorn *et al.*, 1984; Hirata *et al.*, 1984; Irvine *et al.*, 1984; Joseph *et al.*, 1984). Several lines of evidence suggest that, in the β cell, glucose stimulates the metabolism of membrane phosphoinositides (Clements & Rhoten, 1976; Laychock, 1983; Dunlop & Larkins, 1984), with production of [^{3}H]IP_3 after incorporation of [^{3}H]inositol into membrane phospholipids (Best & Malaisse, 1984). Ca^{2+} mobilization by IP_3 in non-tumour-derived insulin-secreting β cells is here shown by using digitonin-permeabilized islets (Colca *et al.*, 1985).

Abbreviations used: IP_3, *myo*-inositol 1,4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate.

Experimental

Male Sprague–Dawley rats (200–300g) were purchased from Sasco (O'Fallon, MO, U.S.A.). Collagenase (CLS IV) was obtained from Cooper

Biochemical (Freehold, NJ, U.S.A.), $^{45}CaCl_2$ (sp. radioactivity 800mCi/mmol) and ^3H_2O were from New England Nuclear, the calcium ionophore A23187 was from Behring Diagnostics (La Jolla, CA, U.S.A.), and sodium vanadate was from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.); other chemicals, except as specified, were from Sigma.

Pancreatic islets were obtained after collagenase digestion and separation on a discontinuous Ficoll gradient (McDaniel *et al.*, 1983). Islets were then permeabilized with digitonin (Colca *et al.*, 1985).

Permeabilized islets (30/100μl assay volume) were first loaded with 5μCi of ^{45}Ca and with 5μCi of ^3H_2O as a cell volume marker for 30min at room temperature in a Tris buffer [50mM-Tris/100mM-KCl / 7mM-$MgCl_2$ / 5mM-ATP / 0.2mM-EGTA (0.06μM free Ca^{2+}, pH7.2)]. The buffer also contained 5μg of Ruthenium Red/ml to inhibit mitochondrial uptake. In some Ca^{2+}-efflux studies, vanadate (1.25mM) was used to inhibit the reuptake of $^{45}Ca^{2+}$ by endoplasmic reticulum. ^{45}Ca content was expressed as pmol of Ca^{2+}/islet after correcting for cell volume and subtracting non-ATP-dependent Ca^{2+} diffusion.

Free Ca^{2+} concentrations were determined by using an apparent stability constant for Ca^{2+}–EGTA of $10^{6.85}$ (24°C, 100mM-KCl, pH7.2) which was determined from an absolute stability constant of $10^{10.97}$ (20°C, I0.1M) by interpolating for temperature and the H^+ activity coefficient (Martell & Smith, 1974).

IP_3 and IP_2 were prepared from human polycythaemic red blood cells not over 2 weeks in age by using the method of Downes & Michell (1981) with modifications as follows. Ghosts were prepared from rejuvenated red cells (Shukla *et al.*, 1979) followed by washing in a continuous-flow filtration apparatus (Pellicon Cassette System; Millipore Corp., Bedford, MA, U.S.A.) (Rosenberry *et al.*, 1981). Ghosts from 6 units of blood were combined and treated with 2mM-Ca^{2+} (37°C, 45min) to release inositol phosphates from the phosphoinositides. The ghosts were then removed with the Pellicon system. The IP_2 and IP_3 were obtained from the filtrate (Downes & Michell, 1981), analysed by electrophoresis (Seiffert & Agranoff, 1965) and by g.l.c. (Sherman *et al.*, 1971; Agranoff & Seguin, 1974). Yields were approx. 6μmol of IP_2 and 15μmol of IP_3/6 units of blood. D-Inositol 1-phosphate and DL-inositol cyclic 1,2-phosphate were prepared as described (Grado & Ballou, 1961; Sherman *et al.*, 1981).

Results

Characterization of Ca^{2+} uptake by the endoplasmic reticulum of digitonin-permeabilized islets showed that this was an ATP-dependent process with maximal uptake occurring at 5mM-ATP (range tested 0–10mM; results not shown). This process was rapid; steady state occured within the first 10min and remained stable for 1h (Fig. 1). The calcium ionophore A23187 (2μM) induced a nearly total reversal of ATP-dependent Ca^{2+} uptake within 5 or 10min (Fig. 1). Vanadate, a Ca^{2+}-ATPase inhibitor (Bond & Hudgins, 1980), was used during the efflux studies in order to

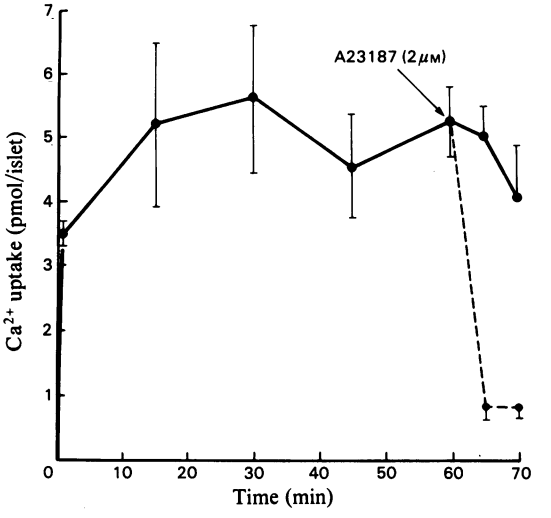

Fig. 1. *Time course of ATP-dependent Ca^{2+} uptake by digitonin-treated pancreatic islets*

Isolated islets were permeabilized with digitonin. In brief, islets were incubated for 20min at 37°C in a modified Krebs buffer [115mM-NaCl/5mM-KCl/24mM-$NaHCO_3$/1mM-$MgCl_2$/25mM-Hepes (pH6.8)/1mM-EGTA/0.1% bovine serum albumin] with digitonin (20μg/ml) and then washed three times in Tris buffer [50mM-Tris (pH6.8)/100mM-KCl/5mM-$MgCl_2$/0.1% bovine serum albumin]. Permeabilized islets (30/tube) were then loaded for the indicated time (0–60min) with 5μCi of ^{45}Ca, and with 5μCi of ^3H_2O as a cell volume marker, at room temperature in a buffer mimicking intracellular conditions [50mM-Tris (pH6.8)/100mM-KCl/7mM-$MgCl_2$/5mM-ATP/100μM-$CaCl_2$] containing 5μg of Ruthenium Red/ml). After 60min loading time, efflux induced by calcium ionophore A23187 (2μM) was measured after 5 and 10min with ethanol as control (final concn. 5%). ^{45}Ca content of the endoplasmic reticulum was determined by centrifugation at 10000*g* for 1min, aspiration of the supernatant, followed by dissolution of the islet pellet with 50μl of Protosol and neutralization with 10μl of acetic acid. Non-ATP-dependent Ca^{2+} uptake was determined separately for each experiment and, at each time, was subtracted from the data. Results are expressed as the means ± S.E.M. of ATP-dependent Ca^{2+} uptake (pmol/islet) for three experiments performed in triplicate.

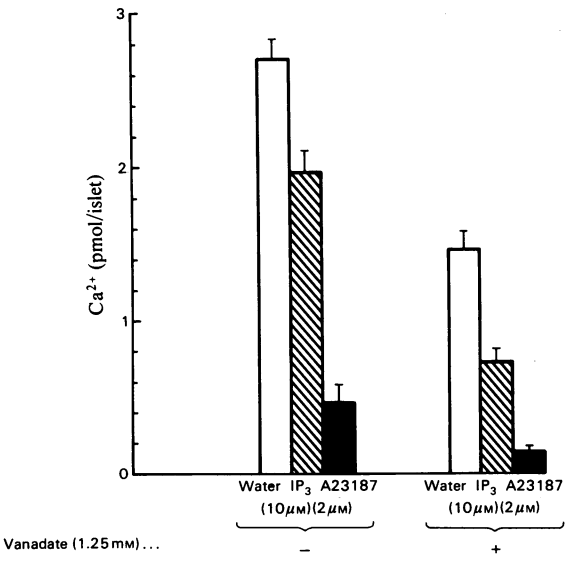

Fig. 2. *Effect of IP_3 on Ca^{2+} efflux (10 min) by digitonin-treated islets*

Isolated islets were permeabilized with digitonin and loaded with 5 μCi of ^{45}Ca and 5 μCi of ^3H_2O as a cell volume marker for 30 min at room temperature in a Tris buffer mimicking intracellular conditions [50 mM-Tris (pH 7.2) 100 mM-KCl/7 mM-$MgCl_2$/5 mM-ATP/0.2 mM-EGTA (0.06 μM free Ca^{2+})] containing 5 μg of Ruthenium Red/ml. Ca^{2+} efflux was then measured after 10 min in the presence or absence (water) of 10 μM-IP_3 with or without 1.25 mM-vanadate. Non-ATP-dependent Ca^{2+} uptake determined individually for each experiment was subtracted from the data. A23187 blanks were also subtracted from the relevant data. Results are shown as the means ± S.E.M. of ATP-dependent Ca^{2+} content (pmol/islet). Each group is the mean of 18 observations in three experiments.

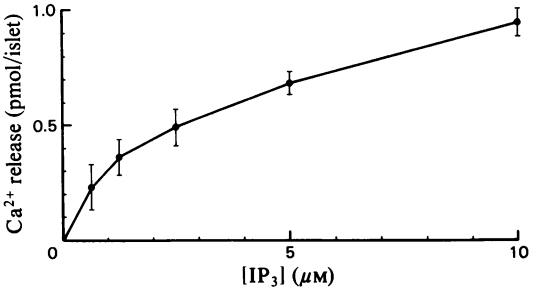

Fig. 3. *Dose–response curve of IP_3-induced Ca^{2+} release by digitonin-treated islets*

Digitonin-permeabilized islets were loaded with Ca^{2+} as described for Fig. 2 and efflux was measured after 10 min in the presence of increasing concentrations of IP_3 (0–10 μM) and 1.25 mM-vanadate. Non-ATP-dependent Ca^{2+} uptake, determined individually for each experiment, was subtracted from the data. Results are expressed as the means ± S.E.M. of Ca^{2+} released (pmol/islet) from 18 observations from three experiments.

minimize Ca^{2+} reuptake by the endoplasmic reticulum. The dose curve of vanadate inhibition of Ca^{2+} uptake showed that 50% inhibition was obtained for vanadate concentrations greater than 0.5 mM (range tested 0–5 mM; results not shown). The effects of efflux agents such as the calcium ionophore A23187 (2 μM) and EGTA (1 mM) were tested after 30 min loading time. Following 5 min exposure to these agents the percentage of Ca^{2+} released was similar with or without vanadate present (1.25 mM), indicating that vanadate did not interfere with efflux, although it did lower steady-state equilibrium level of Ca^{2+}.

The effect of IP_3 on Ca^{2+} efflux was studied at a free Ca^{2+} concentration of 0.06 μM, which is approximately half-maximal for Ca^{2+}-specific insulin secretion under these conditions (Colca *et al.*, 1985). As shown in Fig. 2, 10 μM-IP_3 induced a 30% release ($P<0.001$) of Ca^{2+} from permeabilized islets within 10 min. When 1.25 mM-vanadate was added during the efflux period to minimize Ca^{2+} reuptake, 10 μM-IP_3 induced a 50% efflux ($P<0.001$) of Ca^{2+}, suggesting that vanadate was effectively inhibiting Ca^{2+} reuptake by the endoplasmic reticulum. The phosphoinositide metabolites IP_2 (10 μM), D-inositol 1-phosphate, DL-inositol cyclic 1,2-phosphate and *myo*-inositol (10 and 50 μM) had no significant effect on Ca^{2+} efflux with or without vanadate (results not shown; $n = 9$–18) while IP_2 at 50 μM had a small Ca^{2+}-releasing effect. The dose curve of IP_3 on Ca^{2+} efflux showed dose dependency, with half-maximal release occurring at 2.5 μM-IP_3 and maximal Ca^{2+} release at 10 μM-IP_3 (Fig. 3). Time course studies of IP_3-induced Ca^{2+} release are detailed in Fig. 4. IP_3 provoked maximal efflux of Ca^{2+} from the endoplasmic reticulum by 2 min, followed by slow reuptake. In the presence of vanadate, reuptake was diminished, suggesting that Ca^{2+} released by IP_3 from the endoplasmic reticulum is normally taken up by these same vesicles.

Discussion

The digitonin-permeabilized islet model was used to study Ca^{2+} efflux by the endoplasmic reticulum in the presence of Ruthenium Red which inhibits mitochondrial uptake. No further effect of IP_3 on Ca^{2+} release from the mitochondria was detected in the absence of Ruthenium Red. These data confirm that, in the pancreatic islet-cell, Ca^{2+} uptake by the endoplasmic reticulum is a rapid

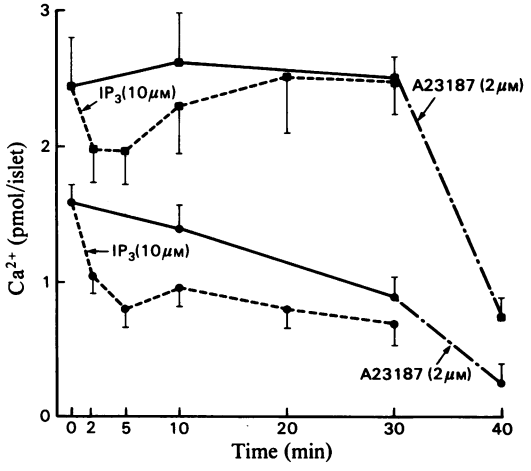

Fig. 4. *Time course of IP_3-induced Ca^{2+} efflux by digitonin-treated islets*

Digitonin-permeabilized islets were loaded with ^{45}Ca as described for Fig. 2 and efflux induced by IP_3 (10μM) was measured after 2, 5, 10, 20 and 30min without (■) or with (●) 1.25mM-vanadate. Non-ATP-dependent Ca^{2+} uptake determined individually for each experiment was subtracted from the data. A23187 blanks were also subtracted from the relevant data. Results are expressed as the means ± S.E.M. of Ca^{2+} content (pmol/islet) from three experiments in triplicate.

ATP-dependent process, reversed in the presence of the Ca^{2+} ionophore A23187 (Colca *et al.*, 1982). The validity of this permeabilized model for the study of insulin secretion as well as Ca^{2+} and calmodulin-dependent protein phosphorylation has also been reported (Colca *et al.*, 1985).

IP_3 caused specific Ca^{2+} release from the endoplasmic reticulum of islet cells. This effect was shown to be dependent on the intracellular Ca^{2+} concentration (levels tested 0.06μM, 0.5μM and 100μM): Ca^{2+} release could only be elicited at a Ca^{2+} concentration in the submicromolar range. This is a range similar to that for Ca^{2+}-induced insulin secretion (Colca *et al.*, 1985). The general characteristics of IP_3-induced Ca^{2+} release from the endoplasmic reticulum of the pancreatic β cell are similar to those described for other tissues, i.e. rapid release that is followed by slow reuptake, although the IP_3 requirements differed among these tissues (see reviews by Berridge, 1984, and Berridge & Irvine, 1984). Our study gives further support for the role of IP_3 as a widely distributed second messenger for agonist-induced intracellular Ca^{2+} mobilization (Berridge, 1984; Berridge & Irvine, 1984).

Several groups have reported that glucose alters the metabolism of pancreatic islet phosphoinositides (Clements & Rhoten, 1976; Laychock, 1983; Dunlop & Larkins, 1984) with production of IP_3 (Best & Malaisse, 1984). The results reported here, taken with our previous study (Colca *et al.*, 1985), suggest that glucose-induced production of IP_3 leads to increased intracellular Ca^{2+} concentrations which are part of the sequence of events that lead to insulin secretion (see review by McDaniel *et al.*, 1984).

In the presence of vanadate, IP_3-induced Ca^{2+} release was increased to 50%, in comparison with 30% in the absence of vanadate. Thus, the reuptake component of IP_3-induced Ca^{2+} release is probably associated with the β cell endoplasmic reticulum Ca^{2+}-ATPase (Colca *et al.*, 1983*b*).

In conclusion, our results support a role for IP_3 as a second messenger for Ca^{2+} release from the endoplasmic reticulum of the β cell, and are consistent with an intracellular link between Ca^{2+} mobilization and insulin secretion.

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