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Studies on the role of inositol trisphosphate in the regulation of insulin secretion from isolated rat islets of Langerhans

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Glucose (20mM) and carbachol (1mM) produced a rapid increase in [^{3}H]inositol trisphosphate ($InsP_3$) formation in isolated rat islets of Langerhans prelabelled with *myo*-[^{3}H]inositol. The magnitude of the increase in $InsP_3$ formation was similar when either agent was used alone and was additive when they were used together. In islets prelabelled with $^{45}Ca^{2+}$ and treated with carbachol (1mM), the rise in $InsP_3$ correlated with a rapid, transient, release of $^{45}Ca^{2+}$ from the cells, consistent with mobilization of $^{45}Ca^{2+}$ from an intracellular pool. Under these conditions, however, insulin secretion was not increased. In contrast, islets prelabelled with $^{45}Ca^{2+}$ and exposed to 20mM-glucose exhibited a delayed and decreased $^{45}Ca^{2+}$ efflux, but released 7–8-fold more insulin than did those exposed to carbachol. Depletion of extracellular Ca^{2+} failed to modify the increase in $InsP_3$ elicited by either glucose or carbachol, whereas it selectively inhibited the efflux of $^{45}Ca^{2+}$ induced by glucose in preloaded islets. Under these conditions, however, glucose was still able to induce a small stimulation of the first phase of insulin secretion. These results demonstrate that polyphosphoinositide metabolism, Ca^{2+} mobilization and insulin release can all be dissociated in islet cells, and suggest that glucose and carbachol regulate these parameters by different mechanisms.

Stimulation of insulin secretion from the endocrine pancreas is believed to result from an increase in cytosolic free Ca^{2+} in the islet B-cells (see Wollheim & Sharp, 1981, for a review). It is thought that glucose increases cytoplasmic Ca^{2+} by promoting a net influx of extracellular Ca^{2+} into the B-cell by the gating of voltage-sensitive Ca^+ channels located in the plasma membrane (Dean & Matthews, 1970; Malaisse *et al.*, 1978; Lebrun *et al.*, 1982; Malaisse-Lagae *et al.*, 1984; Morgan & Montague, 1982; Wollheim & Sharp, 1981). In addition, evidence has been presented which suggests that mobilization of Ca^{2+} from intracellular sites may also play a role in the early phase of the secretory response to glucose (Wollheim *et al.*, 1978; Wollheim & Sharp, 1981).

Several studies have revealed that Ca^{2+} mobilization from intracellular pools in a variety of tissues (Berridge, 1984), including insulin-secreting tumour cells (Biden *et al.*, 1984; Prentki *et al.*, 1984*a*), can be induced by $InsP_3$. This molecule is produced as a result of the phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate present in cellular membranes (Hawthorne, 1983). In this context, evidence has been presented which suggests that an early response to glucose stimulation of islet cells is the breakdown of phosphatidylinositol bisphosphate (Laychock, 1983; Dunlop & Larkins, 1984; Montague *et al.*, 1985), with the resultant generation of inositol phosphates (Best & Malaisse, 1983*a*), including $InsP_3$ (Montague *et al.*, 1985). Furthermore, Biden *et al.* (1984) have suggested that increases in B-cell $InsP_3$ may represent the crucial link between the metabolic and cationic events associated with nutrient-stimulated insulin release.

In order to investigate the role of $InsP_3$ in insulin secretion, we have compared the response of islet cells to stimulation with the muscarinic cholinergic agonist carbachol and the nutrient secretagogue glucose, both of which induce changes in polyphosphoinositide metabolism in islet cells (Best & Malaisse, 1983*a*). Our data clearly demonstrate that, in freshly isolated rat islets of Langerhans, increases in $InsP_3$ can be dissociated from cellular $^{45}Ca^{2+}$ efflux and that neither parameter is obligatorily coupled to stimulation of insulin secretion.

Abbreviation used: $InsP_3$, inositol trisphosphate.

Experimental

Materials

The sources of the materials used in this study were as described by Montague *et al.* (1985).

Methods

Isolation and incubation of islets of Langerhans. Islets of Langerhans were isolated from pancreatic tissue obtained from male Wistar rats (250–350g) by collagenase digestion, as described in detail by Montague & Taylor (1968). All incubations were performed at 37°C in a bicarbonate-buffered medium (Gey & Gey, 1936), gassed with O_2/CO_2 (19:1) to pH7.4 and containing glucose (4mM), $CaCl_2$ (2mM) and bovine serum albumin (3mg/ml). This medium was supplemented with test reagents as described for individual experiments.

Inositol lipid studies. Inositol lipid metabolism in islets was studied by the methods described in detail by Montague *et al.* (1985).

Insulin-secretion studies. After isolation, groups of 80–100 islets were transferred into perifusion chambers (Green *et al.*, 1980; Morgan & Montague, 1982) and perifused at a flow rate of 1ml/min for 45min. Test substances were introduced into the influent medium after the stabilization period, and samples of effluent medium were collected and assayed for insulin content by radioimmunoassay as described previously (Morgan & Montague, 1984).

Islet-cell $^{45}Ca^{2+}$ efflux. Groups of 80–100 islets were incubated for 30min in 100μl of gassed (O_2/CO_2, 19:1) medium (37°C) containing glucose (20mM) and $^{45}Ca^{2+}$ (200μCi/ml) but no added $^{40}Ca^{2+}$. After the loading period, the islets were washed with 2 × 500μl of fresh medium containing 2mM-$^{40}Ca^{2+}$ and were transferred to perifusion chambers. The islets were perifused at a flow rate of 1ml/min for 45min before commencement of the experiment. Test substances were then introduced into the medium as described for individual experiments, and samples of the effluent perifusate were collected. Portions of each sample (400μl) were transferred to scintillation vials, and 4ml of Fisofluor 2 scintillant was added, for determination of $^{45}Ca^{2+}$ content by liquid-scintillation spectrometry. The extent of $^{45}Ca^{2+}$ efflux in response to test agents was expressed relative to the control rate averaged over the 5min immediately before their introduction (100%). In experiments where 20mM-glucose was employed as stimulus, samples of effluent medium were also assayed for insulin to establish the temporal relationship between changes in islet-cell $^{45}Ca^{2+}$ efflux and

Table 1. *Time course for the effect of carbachol on inositol phosphate metabolism in rat islets of Langerhans*

Groups of 40 islets were incubated for 90min with [^{3}H]inositol to prelabel their inositol lipids. The radioactive medium was replaced with fresh medium containing 1mM-*myo*-inositol, and 10min later (zero time) carbachol (1mM) was added. The incubation was terminated at the times shown by the addition of acidified methanol, and the inositol phosphates were separated. Each value is the mean ± S.E.M. for six observations.

	Inositol phosphate-associated radioactivity (c.p.m./40 islets)		
Time (s)	Inositol phosphate	Inositol bisphosphate	Inositol trisphosphate
0	4580 ± 145	290 ± 15	360 ± 20
30	5083 ± 190	360 ± 18	450 ± 25
60	5359 ± 250	386 ± 25	490 ± 30
120	5496 ± 200	403 ± 25	518 ± 30

Table 2. *Effect of carbachol on phosphoinositide metabolism in rat islets of Langerhans*

Groups of 40 islets were prelabelled with [^{3}H]inositol and then incubated with fresh medium containing 1mM-*myo*-inositol. After 10min incubation (37°C) carbachol (1mM) was added, and 1min later the reaction was terminated by addition of acid methanol. The inositol-containing phospholipids were extracted and separated and their radioactivity was determined. Each value is the mean ± S.E.M. for nine observations. Levels of significance: *$P < 0.01$ relative to control islets.

	Phosphoinositide-associated radioactivity (c.p.m./40 islets)		
Incubation conditions	Phosphatidyl-inositol	Phosphatidylinositol phosphate	Phosphatidylinositol bisphosphate
4mM-Glucose	9010 ± 200	250 ± 14	218 ± 12
4mM-Glucose + 1mM-carbachol	8100 ± 300*	205 ± 15*	153 ± 10*

insulin secretion. ^{45}Ca loading did not modify the insulin-secretory responses observed.

Results

Effects of glucose and carbachol on islet inositol polyphosphate content

The data presented in Table 1 demonstrate that treatment with 1 mM-carbachol of islets that had been prelabelled with [^{3}H]inositol caused a rapid increase in the formation of [^{3}H]inositol phosphates. The effect was evident within 30s and was essentially maximal within 1 min (Table 1). This increase was accompanied by a corresponding loss of label from inositol-containing phospholipids (Table 2), suggesting that the response reflected the receptor-mediated degradation of phospholipid by phospholipase C. Raising the glucose concentration of the islet incubation medium from 4 to 20 mM also provoked a rapid increase in [^{3}H]inositol phosphate production, the extent of the stimulation being very similar to that observed with 1 mM-carbachol (Table 3). When glucose (20 mM) and carbachol (1 mM) were used in combination, the resultant increase in inositol bisphosphate and InsP_3 production was additive with that seen in response to each agent alone, although inositol phosphate formation was not further enhanced under these conditions (Table 3).

Effects of glucose and carbachol on islet-cell $^{45}Ca^{2+}$ efflux and insulin release

When groups of isolated islets were preloaded with $^{45}Ca^{2+}$ for 30 min and transferred to perifusion chambers, introduction of 1 mM-carbachol into the medium resulted in a rapid efflux of $^{45}Ca^{2+}$ from the cells (Fig. 1). The effect was

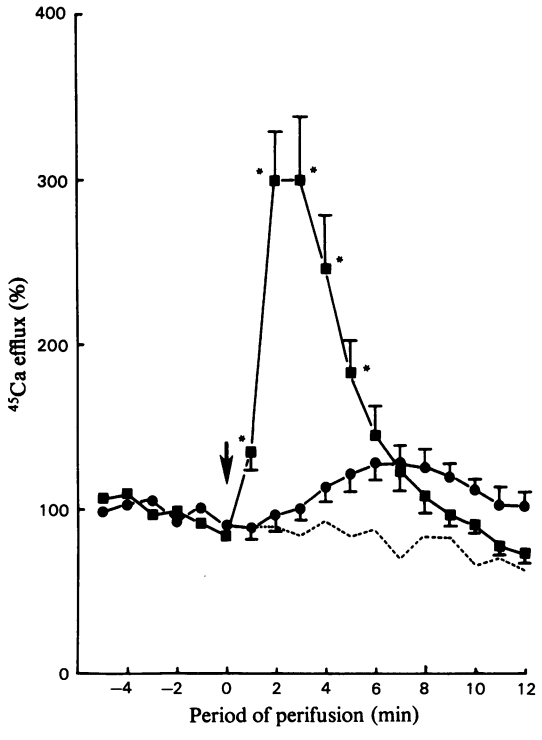

Fig. 1. *Effect of glucose and carbachol on $^{45}Ca^{2+}$ efflux from preloaded islets of Langerhans*

Groups of 80–100 islets were preloaded with $^{45}Ca^{2+}$, then transferred to perifusion chambers. After 45 min of perifusion (zero time) either carbachol (1 mM; ■) or glucose (20 mM; ●) was introduced and samples of perifusate were collected for determination of their $^{45}Ca^{2+}$ content. The broken line represents a typical control perifusion, and each value is the mean ± S.E.M. for four (glucose) or five (carbachol) perifusions. Levels of significance: $*P < 0.001$ relative to 20 mM-glucose.

Table 3. *Effect of glucose and carbachol on inositol phosphate metabolism in rat islets of Langerhans*

Groups of 40 islets were incubated with [^{3}H]inositol to label their inositol lipids. After a preincubation of 10 min in fresh medium, glucose and carbachol were added as shown and the reaction was terminated 1 min later. The inositol phosphates were separated and their radioactivity was determined. Each result is the mean ± S.E.M. for nine observations. Levels of significance: $*P < 0.01$ relative to 4 mM-glucose alone; $**P < 0.01$ relative to 20 mM-glucose and 4 mM-glucose + 1 mM-carbachol.

Incubation conditions	Inositol phosphate-associated radioactivity (c.p.m./40 islets)		
	Inositol phosphate	Inositol bisphosphate	Inositol trisphosphate
4 mM-Glucose	4330 ± 155	296 ± 13	357 ± 18
20 mM-Glucose	5178 ± 200*	385 ± 26*	491 ± 30*
4 mM-Glucose + 1 mM-carbachol	5350 ± 198*	398 ± 28*	478 ± 29*
20 mM-Glucose + 1 mM-carbachol	5637 ± 300	503 ± 35**	657 ± 40**
20 mM-Glucose + 4 mM-EGTA	5494 ± 250*	392 ± 28*	480 ± 29*
4 mM-glucose + 1 mM-carbachol + 4 mM-EGTA	5380 ± 210*	395 ± 18*	480 ± 40*

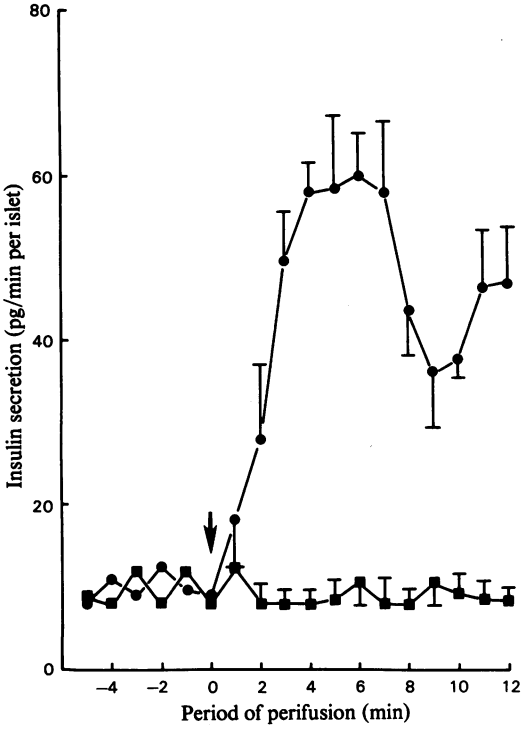

Fig. 2. *Effect of glucose and carbachol on insulin secretion from rat islets of Langerhans*
Groups of 80–100 islets were perifused for 45 min (zero time), when either carbachol (1 mM; ■) or glucose (20 mM; ●) was introduced. Samples of effluent medium were collected and their insulin content was measured. Each point represents the mean value ± S.E.M. for four perifusions.

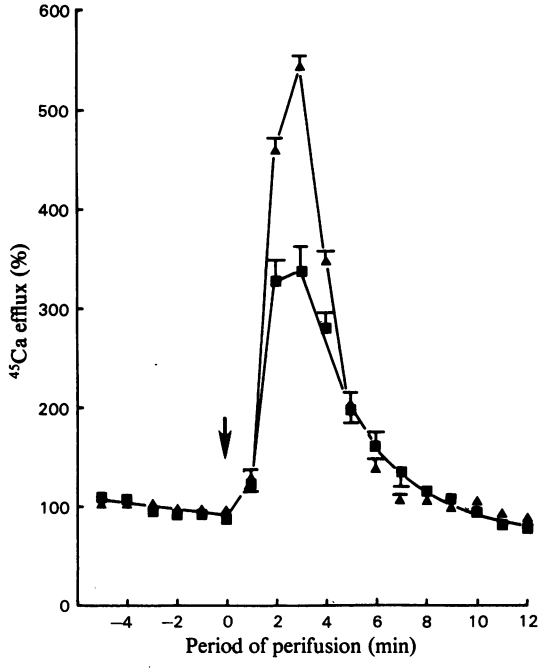

Fig. 3. *Effect of Ca^{2+} depletion on carbachol-induced $^{45}Ca^{2+}$ efflux from isolated rat islets of Langerhans*
Groups of 80–100 islets were preloaded with $^{45}Ca^{2+}$, then transferred to perifusion chambers. Control islets were perifused with medium containing 2 mM-$CaCl_2$ (■), and a second group was exposed to medium containing no added $CaCl_2$ (▲). After 45 min of perifusion (zero time) carbachol (1 mM) was introduced and the $^{45}Ca^{2+}$ content of successive samples of perifusate determined. Each point is the mean value ± S.E.M. obtained from four perifusions.

evident within 1 min of exposure to carbachol and was maximal within 2 min (Fig. 1), when a 3–4-fold increase in $^{45}Ca^{2+}$-efflux rate relative to the control value was observed. Insulin release was not increased under these conditions (Fig. 2). In contrast, when preloaded islets were exposed to 20 mM-glucose a large, biphasic, release of insulin ensued (Fig. 2), which was accompanied by a delayed and rather modest increase in $^{45}Ca^{2+}$ efflux (Fig. 1).

Effect of Ca^{2+} depletion on islet-cell responses to glucose and carbachol

The ability of glucose or carbachol to increase the production of [^{3}H]inositol phosphates in islet cells was unaffected by removal of extracellular Ca^{2+} (Table 3). Similarly, carbachol-induced $^{45}Ca^{2+}$ efflux from preloaded perifused islets was not inhibited when the perifusion medium was depleted of Ca^{2+} (Fig. 3). Indeed, $^{45}Ca^{2+}$ efflux was somewhat enhanced under these conditions (Fig. 3). The modest effect of glucose on $^{45}Ca^{2+}$ efflux was, however, abolished by removal of extracellular Ca^{2+} (Fig. 4).

Effect of glucose on carbachol-induced $^{45}Ca^{2+}$ efflux from preloaded islets

Exposure of preloaded islets to 20 mM-glucose in Ca^{2+}-depleted medium before the introduction of carbachol (Fig. 4) failed to modify the $^{45}Ca^{2+}$-efflux profile induced by the agonist compared with that in the presence of 4 mM-glucose (Fig. 3). Carbachol-induced $^{45}Ca^{2+}$ efflux was, however, abolished when an equimolar concentration of atropine was included in the perifusion medium (results not shown).

Discussion

Current interest has focused on the possible role of $InsP_3$ as a mediator of hormone action in Ca^{2+}-responsive tissues, since this agent appears to mobilize Ca^{2+} from intracellular sites in a variety of cell types (Berridge, 1984). Two recent studies

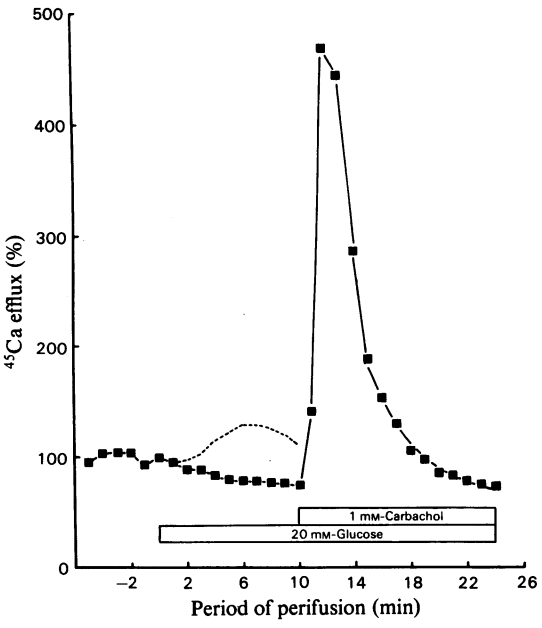

Fig. 4. *Effect of glucose on carbachol-induced $^{45}Ca^{2+}$ efflux from preloaded islets of Langerhans*

Groups of 80–100 islets were preloaded with $^{45}Ca^{2+}$, then transferred to perifusion chambers. After 45min of perifusion with medium containing no added Ca^{2+} (zero time) 20mM-glucose was introduced; 10min later 1mM-carbachol was also infused, and samples were collected for determination of $^{45}Ca^{2+}$ efflux. The data represent mean values from duplicate perifusions. The broken line illustrates the $^{45}Ca^{2+}$-efflux profile obtained with 20mM-glucose in medium containing 2mM-Ca^{2+} (Fig. 1).

(Prentki *et al.*, 1984*a*; Biden *et al.*, 1984) have provided evidence that such a mechanism could be operative in insulin-secreting cells, and our data are consistent with this possibility. Indeed, both glucose and carbachol provoked a substantial increase in InsP_3 formation in islet cells (Tables 1 and 3; Montague *et al.*, 1985), and caused efflux of $^{45}Ca^{2+}$ from preloaded islets (Fig. 1). However, during the course of these experiments, marked discrepancies between the extent of cellular $^{45}Ca^{2+}$ efflux and the rises in InsP_3 in response to these agents became apparent. Carbachol elicited a rapid increase in $^{45}Ca^{2+}$ efflux from preloaded islets, which correlated temporally with the generation of InsP_3 in islet cells (Table 1; Fig. 1). This would be consistent therefore with the possibility that InsP_3 acts to mobilize a Ca^{2+} pool within the cell, with some of the Ca^{2+} then being extruded by the plasma-membrane Ca^{2+}-dependent ATPase (Colca *et al.*, 1983*a*). However, under identical labelling conditions, glucose stimulation elicited a more delayed and much smaller efflux of $^{45}Ca^{2+}$ than did carbachol, despite inducing an equivalent increase in InsP_3 (Fig. 1, Table 3). Furthermore, Ca^{2+} depletion abolished the glucose effect on $^{45}Ca^{2+}$ efflux, but it slightly enhanced the response to carbachol (Fig. 3). Under both of these conditions, however, an increase in cell InsP_3 persisted (Table 3). It appears therefore that efflux of $^{45}Ca^{2+}$ from preloaded islets can be readily dissociated from increases in islet-cell InsP_3.

One possible explanation for these results would be that the extruded $^{45}Ca^{2+}$ did not represent Ca^{2+} released from the InsP_3-sensitive pool(s). It seems likely, however, that, under the conditions of short-term labelling that we used, exchange of $^{45}Ca^{2+}$ would have occurred predominantly within the pool which is responsible for maintaining the lowest set point for intracellular free Ca^{2+}. Under physiological conditions this function appears to be fulfilled by the endoplasmic reticulum in insulin-secreting cells (Biden *et al.*, 1984; Colca *et al.*, 1983*b*; Prentki *et al.*, 1984*b*). Since this also appears to be the InsP_3-sensitive pool (Biden *et al.*, 1984; Prentki *et al.*, 1984*a*), we believe that our measurements of cellular $^{45}Ca^{2+}$ efflux reflect, at least in part, InsP_3-induced mobilization of intracellular Ca^{2+}. This concept is supported by the observation that $^{45}Ca^{2+}$ efflux persisted in the absence of extracellular Ca^{2+} when carbachol was used as agonist (Fig. 3), demonstrating that the $^{45}Ca^{2+}$ must have originated from an intracellular site.

The observation that glucose failed to induce $^{45}Ca^{2+}$ efflux from islets perifused in Ca^{2+}-depleted media (Fig. 4; Wollheim & Sharp, 1981), despite causing a substantial increase in intracellular InsP_3 under these conditions (Table 3), demonstrates that InsP_3 generation can be dissociated from extrusion of $^{45}Ca^{2+}$ from the cells. Since Ca^{2+} extrusion is probably mediated by the calmodulin-dependent activation of a plasma-membrane Ca^{2+}-dependent ATPase (Kotagal *et al.*, 1983), it follows that substantial intracellular mobilization of Ca^{2+} should be accompanied by Ca^{2+} efflux. Rorsman *et al.* (1984) have postulated that glucose may act initially to decrease islet-cell cytosolic free Ca^{2+}, an effect that could result from enhanced mitochondrial Ca^{2+} accumulation (Andersson, 1983). It could be envisaged, therefore, that glucose-induced mobilization of intracellular Ca^{2+} was masked in our experiments by immediate sequestration into mitochondria. The data in Fig. 4 suggests that this was not the case, however, since the subsequent addition of carbachol to islets previously exposed to 20mM-glucose was still able to provoke a large efflux of $^{45}Ca^{2+}$ (Fig. 4). These results demonstrate that the intracellular carbachol-sensitive Ca^{2+} pool remained unaffected by

the prior glucose exposure. These observations suggest that glucose and carbachol may have altered islet-cell polyphosphoinositide metabolism and Ca^{2+} mobilization by different mechanisms. This proposal is supported by the observation that maximal doses of carbachol and glucose (Best & Malaisse, 1983*b*) exerted additive effects on [^{3}H]InsP_3 formation in islet cells (Table 3). This suggests that different phospholipase C enzymes are activated by each agent, which, in turn, implies the existence of discrete pools of phosphatidylinositol bisphosphate, which are sensitive to either receptor activation (carbachol) or metabolic changes (glucose) respectively. The differences in Ca^{2+}-mobilizing ability of glucose and carbachol could then reflect either differential compartmentation of the InsP_3 formed, or differences in the isomeric configuration of the respective InsP_3 types (Irvine *et al.*, 1984), with glucose inducing the production of an isomer which is less potent as a Ca^{2+}-mobilizing agent.

Taken together, therefore, our results indicate that any firm conclusion concerning the possibility that InsP_3 may represent the sole link between metabolic and cationic events involved in the initiation of insulin secretion (Biden *et al.*, 1984) is premature. This is especially true since cholinergic agonists which both increase InsP_3 formation and mobilize islet cell Ca^{2+} pools (Table 1, Fig. 1; see also Nenquin *et al.*, 1984) do not consistently initiate insulin secretion (Fig. 2; Gagerman *et al.*, 1978; Nenquin *et al.*, 1984; but see Malaisse *et al.*, 1967; Trus *et al.*, 1979; Wollheim *et al.*, 1980).

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