Influence of staurosporine on glucose-mediated and glucose-conditioned insulin secretion

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The effect of staurosporine, a putative inhibitor of protein kinase C (PKC), on insulin secretion induced by glucose and 4-methyl-2-oxopentanoate (KIC) was examined. In addition, the effects of staurosporine on the actions of other agonists, for which glucose acts as a conditional modifier, were also examined. At 20 nM, staurosporine caused a marked inhibition of second-phase insulin secretion, whether it was stimulated by 10 mM- or 20 mM-glucose, by 15 mM-KIC, or by carbachol or tolbutamide in islets co-perfused with 7.0 mM-glucose. In each case, the second-phase secretory response was inhibited by 70–85%. In contrast, in all cases there was no effect of staurosporine on the magnitude of the first phase of insulin secretion, nor on the time course of first-phase secretion, except when glucose alone was the secretagogue. With either 10 mM- or 20 mM-glucose, the peak of the first phase of insulin secretion was delayed. Staurosporine does not alter glucose metabolism, or the ability of glucose to activate phosphoinositide hydrolysis or to cause the translocation of α -PKC to the membrane. These findings support the concept that PKC activation plays an important role in fuel-induced or fuel-conditioned insulin secretion.

INTRODUCTION

Although there is increasing evidence that protein kinase C (PKC) plays a role in stimulus–secretion coupling in the β -cell, there is as yet no consensus concerning this role (Henquin *et al.*, 1987; Metz, 1988; Arkhammar *et al.*, 1989; Zawalich & Rasmussen, 1990). In particular, there are conflicting data concerning the role of PKC in glucose-induced secretion, even though high glucose concentrations stimulate phosphoinositide (PI) hydrolysis (Best, 1986; Zawalich & Zawalich, 1988) and the production of diacylglycerol (Peter-Reich *et al.*, 1988), an endogenous activator of PKC (Nishizuka, 1988). In a recent review of these data, Wollheim & Regazzi (1990) concluded that it is most unlikely that PKC plays a major role in mediating the action of glucose and other fuel stimuli on insulin secretion. This conclusion was based in part on the observations of Easom *et al.* (1989). These investigators showed that staurosporine, an inhibitor of PKC, completely blocked phorbol-ester-induced insulin secretion, and inhibited glucose-induced secretion by 60%. Further, in the same study Easom *et al.* (1989) showed that the phorbol ester phorbol 12-myristate 13-acetate (PMA), but not 28 mM-glucose, caused an increase in membrane-associated PKC activity. In follow-up studies, Easom *et al.* (1990) showed that PMA stimulates the phosphorylation of the 80 kDa MARCKS protein, a putative substrate for PKC in many cell types (Blackshear *et al.*, 1986). In addition, they found that staurosporine (100 nM) inhibited phorbol-ester-stimulated MARCKS-protein phosphorylation both in homogenates and in intact islets. This compound, staurosporine, is one of a class of protein kinase inhibitors with a relatively high specificity for PKC (Takahashi *et al.*, 1986), and has been used in a variety of cell types to probe PKC function (King & Rittenhouse, 1989; Taylor *et al.*, 1990; White *et al.*, 1990).

In intact islets, Easom *et al.* (1990) found that 500 μ M-carbachol caused a 2-fold increase in MARCKS-protein

phosphorylation and a 2.6-fold increase in the specific binding of [3 H]phorbol dibutyrate to islets, a measure of PKC association with the plasma membrane. In contrast, 16.5 mM-glucose caused no significant increase in [3 H]phorbol dibutyrate binding, and 28 mM-glucose did not cause a significant phosphorylation of the MARCKS protein in intact islets, even though high glucose caused a 3-fold increase in insulin secretion. However, when 500 μ M-carbachol and 10 mM-glucose were employed together, the second phase of insulin release was increased 30-fold, and the phosphorylation of the MARCKS protein increased 3-fold. Under these conditions, 100 nM-staurosporine caused a significant inhibition (70%) of second-phase insulin secretion. From these data, Easom *et al.* (1990) concluded that the activation of PKC plays an important role in carbachol-induced insulin secretion, but is not a primary determinant of glucose-induced insulin secretion.

In contrast with these data and these views, our work has led us to propose that activation of PKC plays an important role in mediating the second phase of glucose-induced, as well as carbachol-induced, insulin secretion (Zawalich & Rasmussen, 1990). In addition to physiological data supporting this view, we have recently reported that, in freshly isolated perfused rat islets, 20 mM-glucose induces a 30-fold increase in second-phase insulin secretion, accompanied by a 3.8-fold increase in membrane-associated PKC as measured by cell fractionation and Western blotting (Ganesan *et al.*, 1990).

Because of these differing results and conclusions, we undertook an examination of the effects of staurosporine on the patterns of insulin secretion induced either by PMA or glucose alone, or in conjunction with other agonists. Several secretagogues all have the property that they will induce a significant insulin-secretory response in islets incubated in 7.0 mM-glucose, but not in islets incubated in 2.75 mM-glucose. Hence, glucose acts as a conditional modifier in the actions of these other agonists (Rasmussen *et al.*, 1990). The present results

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PI, phosphoinositide; KIC, 4-methyl-2-oxopentanoate (α -ketoisocaproate).

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show that concentrations of staurosporine in the range 10–100 nM cause a marked inhibition of second-phase insulin secretion whether induced by high glucose alone, by 4-methyl-2-oxopentanoate (' α -ketoisocaproate'; KIC), or by moderate glucose in conjunction with other agonists. These results support the concept that PKC activation is an important regulatory event in the β -cell, particularly during the second phase of insulin secretion.

METHODS AND MATERIALS

The detailed methodologies employed to assess insulin output from collagenase-isolated islets have been described previously (Zawalich & Zawalich, 1988). Male Sprague–Dawley rats purchased from Charles River were used in all studies. The animals were fed *ad lib.* and weighed 300–400 g. All animals were treated in a manner which complied with the NIH guidelines for the care and use of laboratory animals. After Nembutal (pentobarbital sodium, 50 mg/kg; Abbott, North Chicago, IL, U.S.A.)-induced anaesthesia, islets were isolated by collagenase digestion and hand-picked, with a glass-loop pipette, under a stereomicroscope. They were free of exocrine contamination. Some islets were directly perfused to monitor insulin release. To monitor PI hydrolysis, other groups of islets were incubated for 2 h in *myo*-[2- 3 H]inositol before being perfused. In these labelling experiments, 10 μ Ci of *myo*-[2- 3 H]inositol (initial sp. radioactivity 16.6–23 Ci/mmol) was added to 250 μ l of medium [gassed with O_2/CO_2 (19:1) and maintained at 37 °C] similar to that used for the perfusion studies. Glucose (2.75 mM) was also present. Groups of 28–35 islets were loaded on to nylon filters and then placed in small glass vials. The radioactive incubation medium (200 μ l) was gently added, the vials were stoppered, and the atmosphere above the islets was aerated with O_2/CO_2 (19:1). After 2 h, the islets were washed with 5 ml of non-radioactive medium. All islets were perfused (flow rate 1 ml/min) with either 2.75 mM- or 7 mM-glucose for at least 30 min to establish stable basal insulin-secretory rates and, where appropriate, [3 H]inositol-efflux rates. When staurosporine was used, it was always present in the perfusion media during this stabilization period. Because staurosporine is dissolved in dimethyl sulphoxide, a similar amount of dimethyl sulphoxide (0.1 ml/100 ml of perfusion medium) was added in control perfusions. After this stabilization period, the islets were subjected to various protocols indicated in the Figure legends. Effluent samples were collected and analysed for insulin content (Albano *et al.*, 1972) with rat insulin as standard (lot 615-D63-12-3; Lilly, Indianapolis, IN, U.S.A.) and, when appropriate, for [3 H]inositol content. Labelled inositol phosphates were measured as previously described (Zawalich & Zawalich, 1988). After extraction with 400 μ l of 20% (w/v) $HClO_4$ plus 400 μ l of incubation medium, neutralization with 0.25–0.28 ml of 6 M-KOH, further addition of 5 ml of water, and centrifugation, the supernatant was applied to columns. These columns were prepared by adding anion-exchange resin (AG1-X8, formate form; Bio-Rad, Richmond, CA, U.S.A.) to Pasteur pipettes to give a length of 3 cm. Further additions to the column included 10 ml of water and 5 ml of 5 mM- $Na_2B_4O_7/60$ mM-sodium formate. The inositol phosphates were eluted by addition of 10 ml of 0.1 M-formic acid/0.2 M-ammonium formate ($InsP_1$), 10 ml of 0.1 M-formic acid/0.4 M-ammonium formate ($InsP_2$) and 10 ml of 0.1 M-formic acid/1 M-ammonium formate ($InsP_3$). Samples (0.4 ml) of the eluate were then analysed for radioactivity contents. Glucose usage rates were measured as described previously (Zawalich *et al.*, 1977) by using [5- 3 H]glucose as tracer. PKC translocation was determined as described previously (Ganesan *et al.*, 1990).

Reagents

Hanks' solution was used for the islet isolation. The perfusion medium consisted of 115 mM-NaCl, 5 mM-KCl, 2.2 mM- $CaCl_2$, 1 mM- $MgCl_2$, 24 mM- $NaHCO_3$ and 0.17 g of BSA/dl. Other compounds were added where indicated, and the solution was gassed with O_2/CO_2 (19:1). The [125 I]-labelled insulin used for the insulin assay, 3H_2O and [5- 3 H]glucose were purchased from New England Nuclear (Boston, MA, U.S.A.) and *myo*-[2- 3 H]inositol was from Amersham (Arlington Heights, IL, U.S.A.). Carbachol, BSA (RIA grade), PMA, formic acid, KIC and the salts used to make the Hanks' solution and perfusion medium were purchased from Sigma (St. Louis, MO, U.S.A.). Staurosporine (dissolved in dimethyl sulphoxide) was purchased from Kyowa Hakko U.S.A. Inc. (New York NY, U.S.A.). Forskolin was obtained from Calbiochem (LaJolla, CA, U.S.A.). Tolbutamide was generously given by the Upjohn Co. (Kalamazoo, MI, U.S.A.). Collagenase (Type P) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.).

Statistics

Statistical significance was determined by Student's *t* test for unpaired data or analysis of variance in conjunction with the Newman–Keuls test for unpaired data; *P* < 0.05 was taken as significant. Values presented in the Figures and results represent means $\pm$ S.E.M. of at least three observations.

RESULTS

Effects of staurosporine on glucose-induced insulin secretion

When freshly isolated islets are perfused with either 10 mM- or 20 mM-glucose, there is a biphasic increase in insulin secretion (Fig. 1). With each glucose concentration, the magnitude of the second phase of secretion is considerably greater than that in the first phase. The magnitude of the second-phase secretion, measured at 35–40 min of stimulation, is 555 ± 66 pg/min per islet in islets perfused with 10 mM-glucose and 1688 ± 213 pg/min

Fig. 1. Influence of staurosporine (0.1 or 1 μ M) on 10 mM- or 20 mM-glucose-induced insulin secretion

Groups of islets were perfused for 30 min with 2.75 mM-glucose alone or 0.1 μ M- (●—●) or 1 μ M- (●---●) staurosporine (Stau). For an additional 40 min they were stimulated with (a) 10 mM-glucose (G_{10}) or (b) 20 mM-glucose (G_{20}). Note change of ordinate scale between panels (a) and (b). Mean $\pm$ selected S.E.M. values are given. This and subsequent perfusion Figures have been corrected for the dead space in the perfusion apparatus, 2.5 ml or 2.5 min with a flow rate of 1 ml/min.

Fig. 2. Staurosporine-induced inhibition of insulin secretion

Composite data from studies using PMA (1 or 5 μ M) or 20 mM-glucose as stimulants are depicted. Insulin release in response to 1 μ M-PMA, 5 μ M-PMA and 20 mM-glucose (second phase) refers to the amount of hormone collected in the perfusate during the final 5 min of the perfusion, or 35–40 min after the onset of stimulation with the agonist. It should be noted that staurosporine was also present during the 30 min stabilization period with 2.75 mM-glucose alone. Thus these values shown here were obtained after islets had been exposed to staurosporine for 65–70 min. First-phase release values refer to the peak release value noted during the initial (0–10) min of stimulation with 20 mM-glucose. Calculated IC_{50} values (concn. giving 50% inhibition) for PMA-induced secretion were of the order of 3–4 nM, a result remarkably similar to the IC_{50} of staurosporine for PKC inactivation (2 nM; Easom *et al.*, 1989). For glucose stimulation, the IC_{50} was on the order of 5–6 nM.

Fig. 3. Influence of staurosporine (1 μ M) on 20 mM-glucose-induced [3 H]inositol efflux rates and insulin secretion

Groups of 28–35 islets were incubated for 2 h in [3 H]inositol to label their phosphoinositides. After washing to remove unincorporated label, they were perfused for 30 min in the presence (●) or absence (○) of 1 μ M-staurosporine (Stauro). For an additional 30 min both groups were stimulated with 20 mM-glucose. Fractional rates of [3 H]inositol efflux (a) and insulin secretion (b) were assessed. Means $\pm$ selected S.E.M. values of at least four experiments are given.

Table 1. Inositol phosphate levels in control and staurosporine-treated islets

Groups of 28–35 islets were incubated for 2 h in a *myo*-[2- 3 H]inositol-containing solution. After washing to remove unincorporated label, they were perfused for 30 min with 2.75 mM-glucose ($G_{2.75}$) and for an additional 30 min with 20 mM-glucose (G_{20}). In some experiments (line 2), staurosporine (Stauro, 1 μ M) was included during the entire 60 min period. In the control experiments, similar amounts of dimethyl sulphoxide were included during the perfusion. Mean values $\pm$ S.E.M. for four experiments are given.

Perfusion protocol (min)	Content (c.p.m./40 islets)		
	InsP ₁	InsP ₂	InsP ₃
1. $G_{2.75} \rightarrow G_{20}$ (30) (30)	1132 $\pm$ 178	398 $\pm$ 57	154 $\pm$ 13
2. $G_{2.75} + \text{Stauro} \rightarrow G_{20} + \text{Stauro}$ (30) (30)	998 $\pm$ 85	393 $\pm$ 22	179 $\pm$ 18

Table 2. Effect of staurosporine (20 or 100 nM) on glucose usage by isolated islets

Three groups of islets were studied. In group 1, islets were perfused for 30 min with 2.75 mM-glucose and for an additional 40 min with 20 mM-glucose (G_{20}). They were then retrieved and glucose usage was assessed by the formation of $^3\text{H}_2\text{O}$ from [^3H]glucose. In groups 2 and 3, the islets were similarly treated except that 20 nM- or 100 nM-staurosporine (Stauro) was included not only during the perfusion but also during the 1 h incubation with labelled glucose. Four experiments were performed under each condition, and mean values $\pm$ S.E.M. are given.

	Glucose usage (pmol/h per islet)
1. G_{20}	180 $\pm$ 17
2. $G_{20} + 20 \text{ nM-Stauro}$	192 $\pm$ 14
3. $G_{20} + 100 \text{ nM-Stauro}$	195 $\pm$ 16

per islet in those perfused with 20 mM-glucose. At each glucose concentration, the insulin-secretory response to glucose was altered by prior exposure of islets to staurosporine, either 1 or 0.1 μ M. The most dramatic effect of staurosporine was on second-phase secretion, and the effects of 0.1 μ M- and 1.0 μ M-staurosporine were essentially the same at both glucose concentrations, i.e. a 75–80% inhibition of second phase secretion. In contrast, staurosporine did not appreciably alter the magnitude of the first phase of secretion induced by 10 mM-glucose, and actually enhanced first-phase secretion in response to 20 mM-glucose. When glucose was the secretagogue, staurosporine caused a slight delay in the time of the maximal rate of first-phase insulin secretion (Fig. 1). For example, peak first-phase secretion in response to 20 mM-glucose alone was reached 3–4 min after exposure, but in the presence of 0.1 μ M-staurosporine peak first-phase secretion did not occur until 6–7 min of exposure. A similar delay in peak first-phase secretion was also seen in response to 10 mM-glucose.

In order to define a minimal effective inhibitory dose of staurosporine, a series of experiments similar in design to those shown in Fig. 1 was carried out with doses of 1, 5, 10, 20 and 100 nM-staurosporine and 20 mM-glucose as the secretagogue. The magnitudes of first and second phases of insulin secretion

Table 3. Influence of staurosporine on PKC distribution

Three groups of islets were studied. One group (line 1) was perfused with 2.75 mM-glucose for 45 min. The second group (line 2) was perfused for 30 min with 2.75 mM-glucose and for an additional 15 min with 10 mM-glucose (G_{10}). The third group (line 3) was also stimulated with 10 mM-glucose for 15 min. However, 100 nM-staurosporine (Stauro) was included in the perfusate for the entire 45 min protocol. After the perfusion, the islets were then sonicated, separated into membrane and cytosol fractions by centrifugation in a Beckman Airfuge [172 kPa (25 lb/in²) for 10 min] and processed by immunoblotting using an antibody to α -PKC. The 80 kDa α -PKC band was quantified on a Visage 2000 system. Results are expressed as $100 \times (\text{optical density of an } \alpha\text{-PKC band in the fraction}) / (\text{total optical density})$, where the total optical density is the sum of the optical density of the α -PKC band in the cytosol fraction plus the optical density of the α -PKC band in the membrane fraction. Data are shown as means $\pm$ S.E.M. ($n =$ at least 4) for each condition; $P < 0.005$ for islets incubated with $G_{2.75}$ alone versus G_{10} with or without staurosporine; G_{10} versus G_{10} + staurosporine, not significant. At the time of retrieval from the perfusion apparatus, insulin output in response to $G_{2.75}$ was 29 ± 12 pg/min per islet; in the response to G_{10} alone, it was 376 ± 71 pg/min per islet; and in response to G_{10} + staurosporine, it was 92 ± 15 pg/min per islet.

	PKC (%)	
	Cytosol	Membrane
1. $G_{2.75}$	81.6 ± 1.5	18.4 ± 1.5
2. G_{10}	44.2 ± 5.5	58.8 ± 5.5
3. G_{10} + Stauro	46.5 ± 3.2	53.5 ± 3.2

Fig. 4. Effect of staurosporine (20 nM) on 15 mM-KIC-induced insulin secretion

Groups of 14–16 islets were perfused for 30 min with 2.75 mM-glucose in the presence (●) or absence (○) of 20 nM-staurosporine (Stauro). At this time they were stimulated with 15 mM-KIC for an additional 40 min. Mean $\pm$ selected S.E.M. values of at least three experiments are given.

Fig. 5. Influence of staurosporine (20 nM) on carbachol (1 mM)-induced insulin secretion

Groups of islets were perfused for 30 min with 7.0 mM-glucose (G_7). Staurosporine (Stauro; 20 nM) was included in one group (●). For an additional 40 min the perfusion was continued with the addition of 1 mM-carbachol. Mean $\pm$ selected S.E.M. values of at least three experiments are depicted.

obtained from these studies are summarized in Fig. 2. The results show that concentrations of staurosporine between 5 and 20 nM have no significant effect on peak first-phase secretion, but a dramatic effect on second-phase release. When measured after 35–40 min of stimulation, concentrations of 5 nM-, 10 nM- and 20 nM-staurosporine inhibit second-phase secretion by 40%, 60% and nearly 70% respectively.

Also shown in Fig. 2 are data illustrating that concentrations of staurosporine in the range 1–10 nM are sufficient to inhibit the insulin-secretory response caused by either 1 μ M- or 5 μ M-PMA. With 20 nM-staurosporine, inhibition of PMA-induced insulin secretion approached 95–100%.

In order to examine the possibility that concentrations of staurosporine which cause such profound inhibition of glucose-induced insulin secretion might alter islet-cell metabolism, the effect of staurosporine on glucose-induced alterations in PI metabolism and on glucose usage were determined. The effects of staurosporine on the fractional rate of [³H]inositol efflux from perfused islets is depicted in Fig. 3, and Table 1 presents measurements of inositol phosphates obtained at the end of the perfusion. The data show that 1 μ M-staurosporine has no effect on [³H]inositol efflux from or on the production of labelled inositol phosphates in isolated islets exposed to 20 mM-glucose. In addition, neither 20 nM- nor 100 nM-staurosporine, concentrations which cause a marked decrease in insulin secretion, inhibits glucose usage by islets (Table 2).

In addition to assessing the effect of staurosporine on PI hydrolysis and glucose metabolism, its effect on PKC translocation was also assessed. Staurosporine (100 nM) does not inhibit the ability of 10 mM-glucose to cause the translocation of α -PKC (see Table 3). Thus, as is known from other studies

Fig. 6. Influence of staurosporine (20 nM) on tolbutamide (200 μ M)-induced insulin secretion

Two groups of islets were perfused for 30 min with 7.0 mM-glucose in the presence (●) or absence (○) of 20 nM-staurosporine (Stauro). For an additional 40 min the perfusion was continued with the further addition of 200 μ M-tolbutamide (TOL). Mean $\pm$ selected S.E.M. values of at least three experiments are presented.

Fig. 7. Influence of staurosporine on 25 mM- K^+ -induced secretion

Two groups of islets were studied. One group, after a 30 min perfusion with 2.75 mM-glucose, was stimulated with 25 mM- K^+ . The second group was perfused for 70 min with 20 nM-staurosporine. For the final 40 min, 25 mM- K^+ was included in the medium together with staurosporine (Stauro). Three experiments were conducted under each condition.

in vitro, staurosporine blocks PKC activity, but not its association with the membrane.

Effect of staurosporine on the actions of other secretagogues

KIC is another fuel that is an effective stimulus for insulin secretion. Addition of 15 mM-KIC to the perfusate induces a biphasic increase in insulin secretion (Fig. 4). Exposure of islets to 20 nM-staurosporine has no effect on the first phase of KIC-induced insulin secretion, but causes a 70% inhibition of second-phase secretion.

There are a number of insulin secretagogues for which the extracellular glucose concentration acts as a conditional modifier of their actions. These agents have little effect on insulin secretion from islets incubated in media containing a low glucose concentration (2.75 mM), but stimulate secretion from islets incubated in a modest glucose concentration (7 mM). These secretagogues include carbachol and tolbutamide. The effect of staurosporine on the action of these agents was examined. Addition of 1 mM-carbachol to a perfusate containing 7 mM-glucose leads to a modest biphasic increase in insulin secretion (Fig. 5). In the presence of 20 nM-staurosporine there is little change in the pattern of first-phase secretion, but an 80% inhibition of second-phase secretion. Likewise, 200 μ M-tolbutamide induces a large biphasic insulin-secretory response in islets perfused with 7 mM-glucose (Fig. 6). First-phase secretion is considerably larger than that seen with 20 mM-glucose (compare data in Fig. 1 with those in Fig. 6). Even so, 20 nM-staurosporine had no significant inhibitory effect on the first phase of tolbutamide-induced insulin secretion, but caused a nearly 80% inhibition of second-phase secretion (Fig. 6).

To examine further the question of whether or not

staurosporine has a significant inhibitory effect on the first phase of insulin secretion, islets were perfused with 2.75 mM-glucose and then exposed to 25 mM- K^+ . This increase in K^+ concentration causes a very large but transient increase in insulin secretion, with only a very small increase in the sustained phase of secretion (Fig. 7). As shown in Fig. 7, 20 nM-staurosporine has no significant effect on the large K^+ -induced first-phase secretion, but staurosporine does abolish the small sustained phase of secretion.

Finally, in previous studies we reported that a combination of tolbutamide (200 μ M), forskolin (10 μ M) and PMA (200 nM) can induce a biphasic increase in insulin secretion similar in magnitude and time course to that induced by high glucose (Zawalich *et al.*, 1984). The effects of 20 nM-staurosporine on the islet response to this combination of pharmacological agents are shown in Fig. 8. A 67% decrease in first-phase insulin secretion and a greater than 90% decrease in second-phase secretion were noted.

DISCUSSION

The present results demonstrate that staurosporine in the concentration range of 5–100 nM causes a marked inhibition of the second phase of insulin secretion from freshly isolated perfused rat islets (Figs. 1, 2, 4–8). This inhibition is in the range of 70–85% with 20 nM-staurosporine in islets stimulated with 10 mM- or 20 mM-glucose (Figs. 1 and 2) or with KIC (Fig. 4), or in islets perfused with 7 mM-glucose and then challenged with 1 mM-carbachol (Fig. 5), 200 μ M-tolbutamide (Fig. 6) or K^+ (Fig. 7). When secretion was stimulated with PMA alone (Fig. 2), or

Fig. 8. Influence of staurosporine on secretion evoked by the combination of forskolin, tolbutamide and the phorbol ester PMA

Two groups of islets were perfused for 70 min with 2.75 mM-glucose in the presence (●) or absence (○) of 20 nM-staurosporine (Stauro). After 30 min, the islets were stimulated with a combination of 10 μ M-forskolin (FOR), 200 μ M-tolbutamide (TOL) and 200 nM-PMA. Three experiments were conducted under each condition.

with a combination of tolbutamide, PMA and forskolin (Fig. 8), second-phase secretion was inhibited by 90–95%. These effects of staurosporine are produced without altering glucose metabolism (Table 2), the glucose-induced increase in PI hydrolysis (Table 1 and Fig. 3) or the glucose-induced translocation of PKC (Table 3).

One of the most interesting aspects of the inhibitory action of staurosporine is that its effect is, under most conditions, largely confined to the second phase of insulin secretion. Under several of the experimental conditions studied (Figs. 1, 2, 4–7) staurosporine caused little or no inhibition of peak first-phase insulin secretion, even though in all cases it inhibited second-phase secretion. When 10–20 mM-glucose was used as the stimulus and 20 nM-staurosporine as the inhibitor (Fig. 2), there was little change in the magnitude of the first phase of insulin secretion. With glucose, the peak of the first phase of insulin secretion was delayed when higher concentrations of staurosporine were employed (Fig. 1). It is not clear why such an effect is observed when glucose is the secretagogue, but not when other agents are employed, particularly when another fuel stimulus, 15 mM-KIC, induces a marked first-phase response (Fig. 4), which is largely unaltered by the presence of staurosporine.

It is of interest that staurosporine also causes a marked inhibition of the insulin-secretory response to a combination of tolbutamide, phorbol ester and forskolin (Fig. 8). This combination of pharmacological agents induces an insulin-secretory response greater than that seen with high glucose (Fig. 1). It was this type of observation which first led us to suggest that PKC plays an important role in mediating the second phase of insulin

secretion (Zawalich *et al.*, 1984). The present results support this view. However, in this case staurosporine did cause a significant inhibition of first-phase secretion. This finding suggests that this combination of agents does not mimic all of the actions of high glucose.

Staurosporine causes a 70–80% inhibition of the second phase of insulin secretion induced by high glucose (Figs. 1 and 2), by KIC (Fig. 4), by carbachol at 7 mM-glucose (Fig. 5) or by tolbutamide at 7 mM-glucose (Fig. 6), but nearly complete inhibition when either PMA alone (Fig. 2) or PMA in combination of forskolin and tolbutamide are employed (Fig. 8). These data support the notion that with the former agonists there is a PKC-independent signalling pathway that accounts for 20–30% of second-phase insulin secretion. Henquin *et al.* (1987) have also suggested that multiple signalling pathways operate to regulate insulin secretion.

A variety of approaches and preparations have been employed to investigate the role of PKC activation in stimulated secretion. There appears to be little disagreement that its activation contributes to cholinergic-induced release. Considerably uncertainty and conflict exist, however, concerning its role in hexose-induced release. In cultured islets, Hii *et al.* (1987) concluded that PKC activation may not play an essential role in glucose-induced secretion. This conclusion was reached on the basis of findings made with control islets, whose responses to high glucose were minimal. For example, their control islets after 20–24 h of culture responded with less than a 2-fold increase in insulin release when stimulated with 20 mM-glucose. Our freshly isolated islets respond with a 20–40-fold increase in insulin-secretory rate. This difference in responsiveness may account, at least in part, for the conclusions reached concerning PKC's involvement in glucose-induced release. In HIT cells, Hughes *et al.* (1990) found that PKC depletion not only blocked acetylcholine-induced release but also decreased glucose-induced release. These findings differ from the report by Hii *et al.* (1987). Several points concerning these depletion studies should be emphasized. First, the culture conditions and media used for these depletion studies may markedly influence the results obtained (Thams *et al.*, 1990). Second, depletion of the enzyme by prolonged phorbol ester exposure is usually incomplete, and the contribution of residual PKC activity in the stimulation of secretion has to be considered. Third, it is clear that the properties of HIT cells and cultured islets differ in significant ways from those of freshly isolated islets.

The possibility that an additional signalling pathway, other than PKC activation, is involved in mediating the second phase of insulin secretion may help to explain the differences in results and conclusions reached on the basis of the present results and those reached by Easom *et al.* (1989, 1990). The major difference in study design between our studies and those of Easom *et al.* (1989, 1990) are in the methods for islet preparation. In this and our previous study on PKC translocation (Ganesan *et al.*, 1990), freshly prepared hand-picked islets were employed. In their studies, Easom *et al.* (1989, 1990) employed islets that were prepared by Ficoll-gradient centrifugation and then cultured overnight. There is a major functional difference in islets prepared by these two methods: a difference in their responsiveness to high glucose. Freshly isolated islets respond to 10 mM-glucose with a 15–20-fold increase, and to 20 mM-glucose with 40–60-fold increases, in second-phase insulin-secretory rate (Fig. 1). This marked enhancement of secretion is similar to that observed with the perfused pancreas preparation: high glucose causes a 40–60-fold increase in insulin-secretory rate (Gerich *et al.*, 1974; Grill *et al.*, 1978; Curry, 1986). In contrast, cultured islets, prepared by Ficoll-gradient centrifugation, display a 3–6-fold increase in the insulin-secretory rate in response to 16.5 mM-glucose (Easom

et al., 1989, 1990). It is noteworthy that 10 mM-glucose and 500 μ M-carbachol have a synergistic effect on insulin secretion as well as on the extent of phosphorylation of the 80 kDa MARCKS protein. Furthermore, 100 nM-staurosporine inhibits the insulin-secretory response to this combination by approx. 65%. The most logical explanation for the failure to observed either significant PKC translocation or MARCKS-protein phosphorylation in response to high glucose alone in cultured islets (Easom *et al.*, 1990) is that the islets have lost a large part of their responsiveness to glucose, and in particular that aspect of the second phase of insulin secretion mediated by the activation of PKC. When freshly isolated islets respond to a glucose challenge (10–20 mM), there is an enhanced translocation of PKC to the membrane, and a marked increase in second-phase insulin secretion. This second phase of the secretory response is inhibited by 70–85% by as little as 20 nM-staurosporine. It is noteworthy that the cultured islets have an insulin-secretory response to glucose similar to that of freshly isolated islets treated with staurosporine. This staurosporine-insensitive component of second-phase insulin secretion may be mediated by a signalling pathway that does not involve PKC activation and that is retained in cultured islets.

The present findings support the concept that PKC activation plays an important role in fuel-induced insulin secretion. In particular, it is a major signalling pathway during the second phase of glucose-induced insulin secretion.

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