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Ca^{2+}-induced insulin secretion from electrically permeabilized islets

Loss of the Ca^{2+}-induced secretory response is accompanied by loss of Ca^{2+}-induced protein phosphorylation

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Increasing the cytosolic Ca^{2+} concentration of electrically permeabilized rat islets of Langerhans caused rapid increases in insulin secretion and in ^{32}P incorporation into islet proteins. However, the secretory responsiveness of permeabilized islets was relatively transient, with insulin secretion approaching basal levels within 20–30 min despite the continued presence of stimulatory concentrations of Ca^{2+}. The loss of Ca^{2+}-induced insulin secretion was accompanied by a marked reduction in Ca^{2+}-dependent protein phosphorylation, but not in cyclic AMP-dependent protein phosphorylation. Similarly, permeabilized islets which were no longer responsive to Ca^{2+} were able to mount appropriate secretory responses to cyclic AMP and to a protein kinase C-activating phorbol ester. These results suggest that prolonged exposure to elevated cytosolic Ca^{2+} concentrations results in a specific desensitization of the secretory mechanism to Ca^{2+}, perhaps as a result of a decrease in Ca^{2+}-dependent kinase activity. Furthermore, these studies suggest that secretory responses of B-cells to cyclic AMP and activators of protein kinase C are not dependent upon the responsiveness of the cells to changes in cytosolic Ca^{2+}.

INTRODUCTION

An influx of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels is thought to be of prime importance in the stimulation of insulin secretion from pancreatic B-cells by physiological secretagogues (reviewed by Prentki & Matschinsky, 1987). Studies using Ca^{2+}-sensitive fluorescent probes have shown that insulin secretagogues cause rapid increases in intracellular Ca^{2+} in B-cells (Grapengiesser *et al.*, 1988; Arkhammar *et al.*, 1989; Wang & McDaniel, 1990), and studies using permeabilized cells have demonstrated that elevations in cytosolic Ca^{2+} alone are sufficient to initiate a secretory response (Yaseen *et al.*, 1982; Tamagawa *et al.*, 1985; Jones *et al.*, 1985). Ca^{2+} and other intracellular regulators of insulin secretion are believed to exert their effects within B-cells by the activation of specific protein kinases and subsequent phosphorylation of substrate proteins (reviewed by Harrison *et al.*, 1984). Thus, cyclic AMP, which activates protein kinase A (PKA), and activators of protein kinase C (PKC) also stimulate insulin secretion, and it has been suggested that these agents operate through a Ca^{2+}-dependent mechanism, either by increasing the intracellular Ca^{2+} concentration or by changing the sensitivity of the secretory mechanism to Ca^{2+} (Henquin, 1985; Jones *et al.*, 1985, 1986; Hughes *et al.*, 1987).

We have previously reported preliminary evidence using perifused, electrically permeabilized islets that increases in intracellular Ca^{2+} cause rapid, dose-related, but not sustained, increases in insulin secretion (Jones *et al.*, 1989). We have now further characterized the secretory responses to raised intracellular Ca^{2+} to determine whether the transience of Ca^{2+}-induced insulin secretion can be related to Ca^{2+}-dependent kinase activity. Our previous studies also demonstrated that Ca^{2+}-induced secretory responses are enhanced by the presence of cyclic AMP (Jones *et al.*, 1986, 1989) or the PKC activator, 4β-phorbol 12-myristate 13-acetate (PMA; Jones *et al.*, 1985, 1989). In the present studies we have investigated whether secretory responses to cyclic AMP and PMA require the activation of Ca^{2+}-sensitive secretory processes.

MATERIALS AND METHODS

Materials

ATP, BSA (fraction V), collagenase (type XI), cyclic AMP, dimethyl sulphoxide (DMSO), EGTA, Hepes, 3-isobutyl-1-methylxanthine (IBMX) and PMA were from Sigma Chemical Co. (Poole, Dorset, U.K.). [γ-^{32}P]ATP (< 3000 Ci/mmol) was from Amersham International. All other chemicals were of analytical grade from BDH (Poole, Dorset, U.K.). Rats (Wistar, 150–200 g) were from Charing Cross Hospital Medical School (London, U.K.).

Islet isolation and permeabilization

Islets of Langerhans were isolated from rat pancreata by collagenase digestion (Bjaaland *et al.*, 1988) and washed (five times, 4 °C) in a buffer ('permeation buffer') containing 140 mM-potassium glutamate, 15 mM-Hepes, 7 mM-$MgSO_4$, 5 mM-ATP, 5 mM-glucose, 1 mM-EGTA and 0.5 mg of BSA/ml, pH 6.6, with $CaCl_2$ added to give a Ca^{2+} concentration of 50 nM. Islets were electrically permeabilized by five exposures to an electric field of 3.4 kV/cm as previously described (Jones *et al.*, 1985), and washed (×2, 4 °C) with permeation buffer before use.

Insulin secretion from permeabilized islets

Perifusions. Groups of 50–100 permeabilized islets were transferred to Millipore Swinnex filter chambers containing 1 μm-pore-size nylon filters and perifused at a flow rate of 1 ml/min with permeation buffer containing either 50 nM- or 10 μM-Ca^{2+}, supplemented in some experiments with PMA (500 nM) and in others with cyclic AMP (500 μM) plus IBMX (100 μM) or IBMX (100 μM) alone. PMA and IBMX were dissolved in DMSO such that the final concentration of DMSO was < 0.1% (v/v), which did not affect insulin secretion in these experiments. Perifusate

Abbreviations used: PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulphoxide; IBMX, 3-isobutyl-1-methylxanthine.

fractions were collected at 2 min intervals and insulin content was measured by radioimmunoassay (Jones *et al.*, 1988). Perifusion temperature was maintained at a constant 37 °C by performing experiments in a temperature-controlled chamber.

Batch incubations. After permeabilization, islets were either preincubated for 1 h (37 °C) in permeation buffer containing 50 nM- or 10 μM-Ca^{2+}, or used immediately. Groups of five islets were incubated in 500 μl of permeation buffer for 30 or 60 min at 37 °C, after which islets were pelleted by brief centrifugation (9000 *g*, 10 s) and samples of supernatant were taken for the measurement of insulin release by radioimmunoassay.

Protein phosphorylation in permeabilized islets

Permeabilized islets were preincubated for 1 h (37 °C) in normal permeation buffer or in modified permeation buffer (no ATP, 2 mM-$MgSO_4$) containing 50 nM- or 10 μM-Ca^{2+}. Groups of 50 islets were then incubated (1 min, 37 °C) in permeation buffer (no BSA) containing 0.3 mM-ATP, 2.3 mM-$MgSO_4$ and [γ-^{32}P]ATP (final specific radioactivity 0.8 Ci/mmol) and supplemented with 10 μM-Ca^{2+} or 500 μM-cyclic AMP. Islets were pelleted by brief centrifugation (9000 *g*, 15 s), the supernatant was discarded and the reaction was terminated by the addition of 20 μl of a buffer containing 10% (w/v) SDS, 5% (v/v) mercaptoethanol, 5% (v/v) glycerol, 250 mM-Tris and 0.01% (w/v) Bromophenol Blue, and boiling for 3 min. Proteins were separated by electrophoresis on a gradient (7–17%) polyacrylamide gel in the presence of SDS and ^{32}P incorporation was detected by autoradiography (Jones *et al.*, 1988). In some experiments, autoradiographic density was quantified by scanning densitometry.

RESULTS

Ca^{2+}-induced insulin secretion from electrically permeabilized islets

Permeabilized islets perifused with a buffer containing a sub-stimulatory concentration of Ca^{2+} (50 nM) showed a steady basal rate of insulin secretion (10.4 ± 1.9 pg/min per islet; mean ± S.E.M. of seven separate experiments). Exposure to a stimulatory concentration of Ca^{2+} (10 μM) produced a rapid stimulation of insulin secretion, which returned to basal rates within 20–30 min despite the continued presence of the stimulatory concentration of Ca^{2+} (Fig. 1*a*). Following this secretory response to Ca^{2+}, a further response to Ca^{2+} could not be evoked after a subsequent 20 min exposure to a sub-stimulatory Ca^{2+} concentration (Fig. 1*b*), nor by alternating between 50 nM- and 10 μM-Ca^{2+} at 10 min intervals (Fig. 1*c*). Fig. 1(*d*) shows that permeabilized islets mounted a similar secretory response to 10 μM-Ca^{2+} even after 60 min of exposure to the sub-stimulatory Ca^{2+} concentration. Plasma membranes of the electrically permeabilized islets did not reseal during the course of these perifusions, as judged by the uptake of Trypan Blue. Fig. 2(*b*) shows that essentially all of the cells of electrically permeabilized islets showed positive staining with Trypan Blue, indicative of compromised plasma membranes, after 95 min of perifusion with buffer containing 10 μM-Ca^{2+}. In contrast, Trypan Blue staining of non-permeabilized islets perifused with permeation buffer (10 μM-Ca^{2+}, 95 min) was confined to a few cells on the periphery of the islet, as demonstrated Fig. 2(*a*).

Effects of cyclic AMP and PMA on Ca^{2+}-insensitive islets

We have previously demonstrated that both cyclic AMP and PMA enhance the initial insulin secretory response to a maximum stimulatory concentration of Ca^{2+} (Jones *et al.*, 1989). We have now extended these observations to measure secretory responses of Ca^{2+}-insensitive islets to cyclic AMP and PMA. Fig. 3 shows the results of experiments in which permeabilized islets exhibited the expected pattern of a rapid secretory response to 10 μM-Ca^{2+}, followed by a loss of responsiveness to Ca^{2+}. However, both PMA (Fig. 3*a*) and cyclic AMP (Fig. 3*b*) stimulated insulin secretion from the Ca^{2+}-insensitive permeabilized islets. Similar patterns of insulin secretion were observed in response to PMA (Fig. 4*a*) and cyclic AMP (Fig. 4*b*) in the presence of a sub-stimulatory concentration of Ca^{2+} (50 nM). In the presence of either 50 nM-Ca^{2+} (Fig. 4) or 10 μM-Ca^{2+} (Fig. 3), secretory

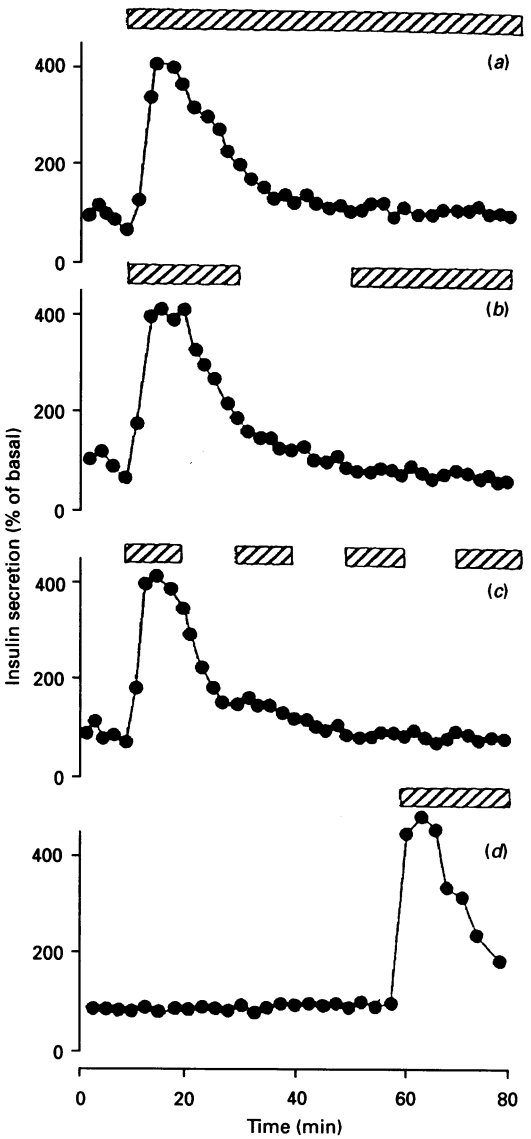

Fig. 1. Ca^{2+}-induced insulin secretion from permeabilized islets

Electrically permeabilized islets were perifused with a buffer containing 50 nM-Ca^{2+} and insulin secretion was stimulated by differing patterns of exposure to a buffer containing 10 μM-Ca^{2+}, as shown by the hatched bars. The loss of secretory responsiveness following exposure to 10 μM-Ca^{2+} (*a*) was not reversed by a subsequent 20 min exposure to 50 nM-Ca^{2+} (*b*), and was seen after 10 min of exposure to 10 μM-Ca^{2+} (*c*). Permeabilized islets retained the ability to respond to 10 μM-Ca^{2+} after a 60 min perifusion with buffers containing 50 nM-Ca^{2+} (*d*). Insulin secretion is expressed as the percentage increase over the basal rate of insulin release (50 nM-Ca^{2+}, 0–10 min). Points show the mean secretion from two separate channels.

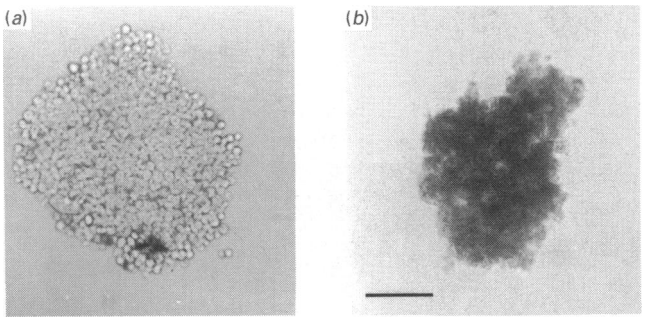

Fig. 2. Trypan Blue staining of permeabilized islets

Trypan Blue staining of an intact islet (*a*) and an electrically permeabilized islet (*b*) after 95 min of perifusion (1 ml/min, 37 °C) with permeation buffer containing 10 μM-Ca^{2+}. Most cells in the intact islet excluded the stain, suggesting that exposing intact islets to the perifusion buffer for 95 min did not increase membrane permeability (*a*). However, electrically permeabilized islets showed extensive staining with Trypan Blue, which is indicative of the compromised plasma membranes of the islet cells (*b*), and suggests that extensive resealing of the plasma membranes had not occurred during the perifusion. The scale bar represents 100 μm.

responses to PMA were considerably slower in onset than those to cyclic AMP, in direct contrast to the rapid effects of both of these agents at a stimulatory concentration of Ca^{2+} (Jones *et al.*, 1989).

Fig. 4 also shows that increasing the Ca^{2+} concentration of the perifusate from 50 nM to 10 μM (at 40–70 min) in the continuing presence of cyclic AMP or PMA produced a rapid, but transient, increase in insulin secretion. This pattern of secretion was similar to that seen in response to 10 μM-Ca^{2+} alone (see Figs. 1 and 3), but was superimposed upon the elevated rates of secretion produced by cyclic AMP or PMA. As observed in other experiments (Figs. 1 and 3), permeabilized islets rapidly became unresponsive to Ca^{2+}, and insulin secretion returned to the plateau cyclic AMP- or PMA-induced rates despite the continued presence of a stimulatory concentration of Ca^{2+}. In control experiments, intact (non-permeabilized) islets showed a much lower rate of insulin secretion than did permeabilized islets when perifused with permeation buffer containing 50 nM-Ca^{2+} (intact islets, 3.2 ± 0.5 pg/min per islet; permeabilized islets, 11.6 ± 1.4 pg/min per islet; means ± S.E.M., $n = 4$). Intact islets showed no increase in insulin secretion when exposed to buffers containing 10 μM-Ca^{2+} (2.5 ± 0.6 pg/min per islet) or 10 μM-Ca^{2+} plus 500 μM-cyclic AMP (3.3 ± 0.5 pg/min per islet), in contrast to the responses of electrically permeabilized islets to elevated Ca^{2+} (Figs. 1 and 2) or cyclic AMP (Figs. 2 and 3).

Table 1. ATP-dependence of insulin secretion from electrically permeabilized islets

Insulin secretion is shown from electrically permeabilized islets during 60 min preincubation periods in buffers containing 50 nM- or 10 μM-Ca^{2+} in the presence or absence of 5 mM-MgATP. Values are means ± S.E.M., $n = 18$. *$P < 0.01$ versus 50 nM-Ca^{2+} in the presence of ATP; †$P < 0.01$ versus 50 nM-Ca^{2+} in the absence of ATP.

	Insulin secretion (pg/h per islet)	
[Ca^{2+}]	No ATP	5 mM-MgATP
50 nM	273 ± 27	359 ± 27†
10 μM	234 ± 19	734 ± 58*

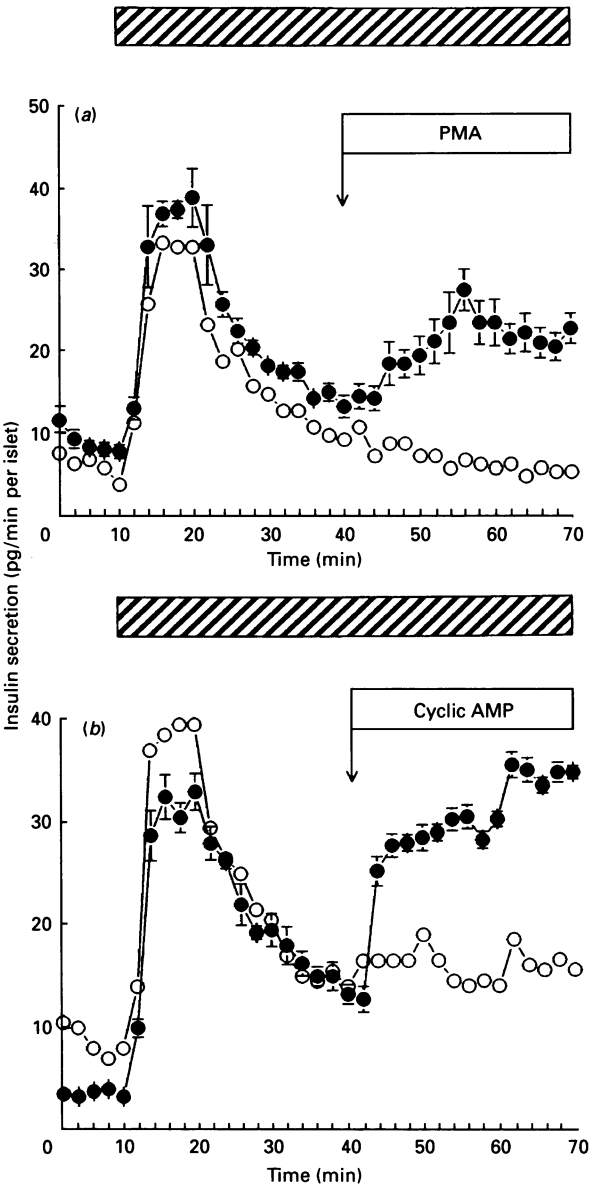

Fig. 3. Cyclic AMP- and PMA-induced secretion from Ca^{2+}-insensitive islets

Permeabilized islets were perifused with a buffer containing 50 nM-Ca^{2+} for 10 min (time, 0–10 min), then exposed to 10 μM-Ca^{2+} for the duration of the experiment, as shown by the hatched bar. After the initial secretory response to 10 μM-Ca^{2+} (at time = 40 min), the islets were exposed to (*a*) PMA (500 nM, ●) or to (*b*) cyclic AMP (500 μM, ●) in the presence of 10 μM-Ca^{2+}. Point show means ± S.E.M. ($n = 4$). Parallel secretory responses to 10 μM-Ca^{2+} alone are shown by ○ (means of two experiments).

Protein phosphorylation in Ca^{2+}-insensitive islets

Loss of secretory responses to Ca^{2+} as a result of prior exposure to stimulatory concentrations of Ca^{2+} (see Figs. 1 and 3) was accompanied by a marked decrease in Ca^{2+}-dependent ^{32}P incorporation into islet proteins. Fig. 5 shows the results of a typical experiment in which permeabilized islets were preincubated (60 min, 37 °C) in the presence of 50 nM- or 10 μM-Ca^{2+}, followed by assessment of ^{32}P incorporation during a subsequent 1 min incubation. Permeabilized islets which had been preincubated for 60 min in the presence of 50 nM-Ca^{2+} retained the ability to respond to 10 μM-Ca^{2+} with increased insulin secretion, as demonstrated in Fig. 6 (see also Fig. 1*d*). Similarly, increasing

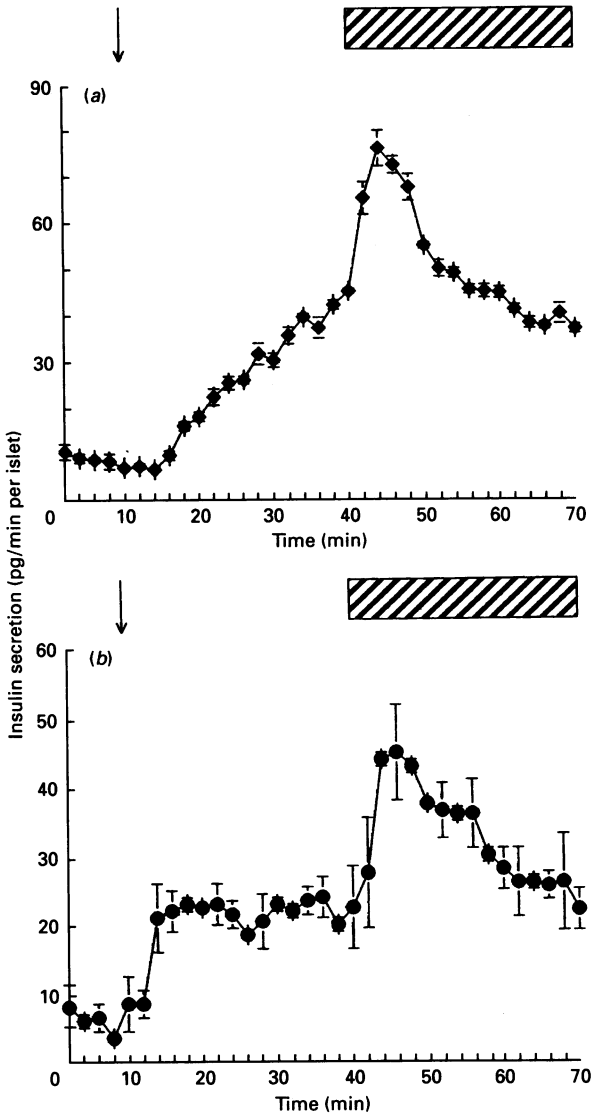

Fig. 4. Cyclic AMP- and PMA-induced secretion from unstimulated permeabilized islets

Permeabilized islets were perifused with a buffer containing 50 nM-Ca^{2+} for 10 min (time 0–10 min) then exposed (arrow) to (*a*) PMA (500 nM; means ± S.E.M., $n = 3$) or to (*b*) cyclic AMP (500 μM; means ± range, $n = 2$) in the presence of 50 nM-Ca^{2+}. After a further 30 min the Ca^{2+} concentration was increased to 10 μM (in the continued presence of cyclic AMP or PMA) for the remainder of the experiment, as shown by the hatched bar.

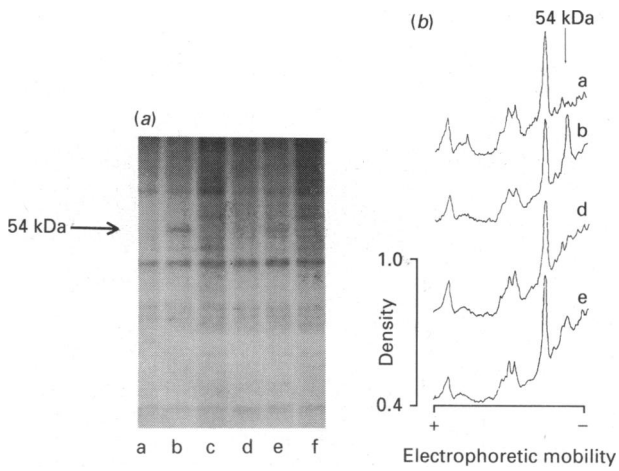

Fig. 5. Protein phosphorylation in permeabilized islets

Permeabilized islets were preincubated (60 min, 37 °C) in buffers containing either 50 nM-Ca^{2+} (a, b and c) or 10 μM-Ca^{2+} (d, e and f). ^{32}P incorporation from [γ-^{32}P]ATP into islet proteins was then assessed following a subsequent incubation (1 min, 37 °C) in the presence of 50 nM-Ca^{2+} (a and d), 10 μM-Ca^{2+} (b and e) or 50 nM-Ca^{2+} plus 500 μM-cyclic AMP (c and f). (*a*) Autoradiography of ^{32}P incorporation into islet proteins separated by PAGE, as described in the Materials and methods section. (*b*) Scanning densitometer traces of lanes a, b, d and e clearly show the Ca^{2+}-induced increase in radiolabel incorporation into the 54 kDa protein in 50 nM-Ca^{2+}-pretreated islets (trace a versus trace b), and the marked decrease in this effect in islets which had been pretreated with 10 μM-Ca^{2+} (trace d versus trace e).

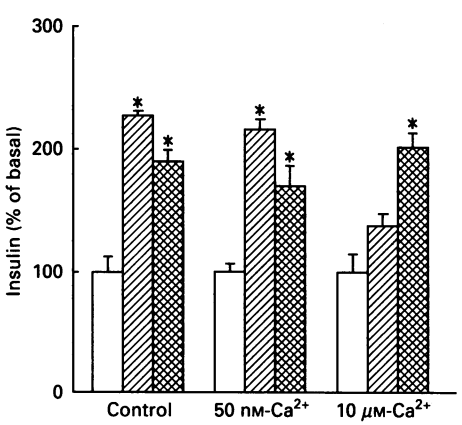

Fig. 6. Insulin secretion from Ca^{2+}-insensitive islets

Shown is insulin secretion from permeabilized islets used immediately after permeabilization (control), or after preincubation (60 min, 37 °C) in buffers containing 50 nM-Ca^{2+} or 10 μM-Ca^{2+}. Preincubation in 50 nM-Ca^{2+} did not affect subsequent secretory responses to elevated Ca^{2+} (10 μM, ▨) or to cyclic AMP (500 μM, ⊠). However, islets which had been preincubated in the presence of 10 μM-Ca^{2+} lost their secretory responsiveness to Ca^{2+}, but not to cyclic AMP. Results are expressed as the percentage increase in insulin secretion over basal release in the presence of 50 nM-Ca^{2+} (□). Bars show means ± S.E.M.; $n = 5$ or 6. $^*P < 0.01$ versus secretion in appropriate 50 nM-Ca^{2+} control.

the intracellular Ca^{2+} concentration to 10 μM caused rapid (1 min) increases in ^{32}P incorporation into several proteins in electrically permeabilized islets which had been preincubated in the presence of 50 nM-Ca^{2+}. The major substrate for Ca^{2+}-dependent phosphorylation in permeabilized islets was a protein of apparent molecular mass 54 kDa, as shown in Fig. 5 (a versus b). However, this Ca^{2+}-dependent phosphorylation was greatly reduced in permeabilized islets which had been preincubated in the presence of 10 μM-Ca^{2+} (Fig. 5, d versus e), and which therefore no longer showed secretory responses to Ca^{2+} (Fig. 6). Cyclic AMP-induced (500 μM) phosphorylation of several proteins was readily detected (Fig. 5, c), and was not affected by preincubation in 10 μM-Ca^{2+} (Fig. 5, f). Similarly, the stimulation of insulin secretion by cyclic AMP was not affected by a 60 min preincubation in buffers containing either 50 nM- or 10 μM-Ca^{2+} (Fig. 6).

Insulin secretion from electrically permeabilized islets was ATP-dependent, as shown in Table 1. Measurement of insulin release from permeabilized islets during the 60 min preincubation period demonstrated that, in the absence of MgATP, basal (50 nM-Ca^{2+}) insulin secretion was decreased, and increasing the Ca^{2+} concentration to 10 μM did not stimulate secretion. How-

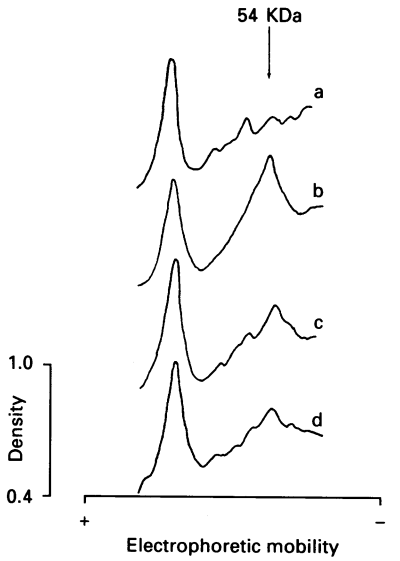

Fig. 7. Ca^{2+}-induced decreases in Ca^{2+}-dependent phosphorylation do not require ATP

Scanning densitometer traces are shown of autoradiographs of ^{32}P incorporation into islet proteins after incubation (1 min, 37 °C) in buffers containing 50 nM-Ca^{2+} (trace a) or 10 μM-Ca^{2+} (traces b, c and d). The permeabilized islets used for traces a and b had been preincubated (60 min, 37 °C) in the presence of 50 nM-Ca^{2+}, and the subsequent exposure to 10 μM-Ca^{2+} (trace b) caused the expected large increase in radiolabel incorporation into the 54 kDa protein (arrow). Preincubation in the presence of 10 μM-Ca^{2+} for 60 min at 37 °C (traces c and d) produced marked decreases in this Ca^{2+}-induced radiolabel incorporation, irrespective of whether the preincubation buffer contained MgATP (5 mM, trace c) or not (trace d).

ever, the loss of Ca^{2+}-dependent ^{32}P incorporation after exposure to 10 μM-Ca^{2+} was not dependent upon the presence of ATP in the buffer during the preincubation period. The scanning densitometer traces shown in Fig. 7 demonstrate that a marked reduction in Ca^{2+}-dependent phosphorylation was seen in both the absence and the presence of 5 mM-MgATP in the preincubation buffer. Since ATP was not required for the development of Ca^{2+}-insensitivity (Fig. 7), it was routinely omitted from the buffers used for the preincubation period to avoid the Ca^{2+}-dependent phosphorylation of substrates with non-radiolabelled phosphate during the preincubation period, which could produce a misleading decrease in ^{32}P incorporation during the subsequent incubation with [γ-^{32}P]ATP.

DISCUSSION

Pancreatic B-cells respond to elevated concentrations of nutrient secretagogues, such as glucose, with increases in their rate of insulin secretion which are sustained for the duration of the stimulus (reviewed by Hedeskov, 1980). The increased rate of insulin secretion is thought to be caused, at least in part, by elevations in cytosolic Ca^{2+}. Direct measurements using fluorescent probes have demonstrated that maximal stimulatory glucose concentrations, which produce pronounced and prolonged secretory responses, also cause sustained elevations in B-cell Ca^{2+} (Arkhammar *et al.*, 1989; Grapengiesser *et al.*, 1989; Wang & McDaniel, 1990; Pralong *et al.*, 1990).

In view of the sustained increases in cytosolic Ca^{2+} in response to physiological secretagogues, the transient secretory responsiveness of electrically permeabilized islets to elevated intracellular Ca^{2+} is somewhat unexpected. There are a number of reasons to suggest that this response reflects a specific desensitization of the secretory mechanism to Ca^{2+}. Thus the loss of responsiveness to Ca^{2+} was not caused by rapid resealing of the electrically permeabilized plasma membranes, since (1) permeabilized islets responded to elevated Ca^{2+} after prolonged exposures to a substimulatory concentration of Ca^{2+}; (2) Ca^{2+}-insensitive islets responded normally to membrane-impermeant cyclic AMP, whereas non-permeabilized islets did not show any such response; (3) [γ-^{32}P]ATP, which will not readily enter intact cells, rapidly entered the Ca^{2+}-insensitive permeabilized islets; and (4) Trypan Blue exclusion experiments showed no evidence of membrane resealing over the time course of the perifusion experiments. Furthermore, the loss of response to elevated Ca^{2+} cannot be ascribed to a general decrease in the exocytotic capacity of permeabilized B-cells caused, for example, by the time-dependent leakage or depletion of an essential cytosolic factor. Thus cyclic AMP and PMA produced a sustained stimulation of insulin secretion over the time course of these experiments, and both cyclic AMP and PMA were able to stimulate secretion from islets which were no longer responsive to Ca^{2+}.

A loss of sensitivity to Ca^{2+} has also been observed in electrically permeabilized chromaffin cells (Knight & Baker, 1982; Knight *et al.*, 1989), although in this cell type it was reported that a return of Ca^{2+}-responsiveness could be obtained by lowering the Ca^{2+} concentration to resting levels for a few minutes. This was clearly not the case for electrically permeabilized islets in the present study, since even a brief exposure (10 min) to elevated Ca^{2+} produced a Ca^{2+}-insensitivity which could not be reversed by lowering intracellular Ca^{2+} to a substimulatory concentration. It is worth noting that Ca^{2+}-induced loss of responsiveness to Ca^{2+} does not appear to be a peculiarity of the electrical permeabilization method, since similar transient responses to Ca^{2+} have been reported for luteinizing-hormone secretion from gonadotropes permeabilized by α toxin from *Staphylococcus aureus* (Van der Merwe *et al.*, 1990). Although no information was provided about the reversibility of Ca^{2+}-insensitivity in these cells, the effect appeared to be specific for Ca^{2+}-induced secretion, since PMA was able to stimulate luteinizing-hormone release from Ca^{2+}-insensitive gonadotropes, similar to our results using PMA and cyclic AMP in Ca^{2+}-insensitive islets. Studies using intact islets also suggest a transient responsiveness of B-cells to elevated Ca^{2+}. Thus Ca^{2+} ionophores produced a transient rather than a sustained release of insulin, and secretion could be maintained by the presence of PMA (Zawalich *et al.*, 1983).

The activation of intracellular protein kinases is thought to be an important step in stimulus–secretion coupling. In the present study, we have demonstrated that increasing the intracellular Ca^{2+} concentration in electrically permeabilized islets causes the rapid phosphorylation of a protein of molecular mass approx. 54 kDa, confirming our previous observations in this model (Jones *et al.*, 1988). Although it is not certain that the 54 kDa substrate is involved in the secretory process, the rapidity of the Ca^{2+}-induced phosphorylation is consistent with the rapid effects of elevated Ca^{2+} on insulin secretion in the present studies. The Ca^{2+}-dependent phosphorylation of a substrate of similar molecular mass has also been reported in studies using intact or homogenized islet tissue (see Harrison *et al.*, 1984), or digitonin-permeabilized islets (Colca *et al.*, 1985). The precise identity and functions of this substrate are not yet clear, although it may be a subunit of tubulin (Colca *et al.*, 1983) or an autophosphorylated Ca^{2+}-dependent kinase (Harrison *et al.*, 1984). Whatever the identity of the 54 kDa islet protein, it is clearly a substrate for a Ca^{2+}-dependent kinase, and the results of the present study

suggest that the phosphorylation of this substrate is much reduced in Ca^{2+}-insensitive islets.

The decrease in ^{32}P incorporation into the 54 kDa protein is unlikely to reflect Ca^{2+}-dependent, non-specific proteolytic degradation (Susuki *et al.*, 1987) or phosphatase activation (Cohen, 1990), since it was not accompanied by decreases in cyclic AMP-dependent ^{32}P incorporation, nor in secretory responses to cyclic AMP or PMA. The most likely explanation for the present results is a Ca^{2+}-induced decrease in Ca^{2+}-dependent kinase activity alone. The mechanism underlying this effect has not yet been identified, but it is clear from our experiments in which islets were preincubated in the absence of ATP that the process of Ca^{2+}-desensitization is not ATP-dependent and so cannot be attributed to autophosphorylation of the Ca^{2+}-dependent kinase. Since the loss of Ca^{2+}-dependent protein phosphorylation seen in the present studies was accompanied by a specific loss of Ca^{2+}-induced insulin secretion, we suggest that the secretory response of permeabilized islets to Ca^{2+} is transient because of the rapid loss of essential Ca^{2+}-dependent kinase activity.

The physiological significance of the Ca^{2+}-induced loss of Ca^{2+} sensitivity is still uncertain, since it is difficult to directly compare secretory responses of permeabilized islets with secretion from intact islets in response to physiological secretagogues. Nonetheless, electrically permeabilized islets offer a useful model for studies of exocytosis, and our experiments with Ca^{2+}-insensitive islets allow us to draw some conclusions about interactions between Ca^{2+} and other intracellular regulators of secretion. In intact insulin-secreting tumour cells, PMA or the adenylate cyclase activator forskolin reduced the requirement of the secretory mechanism for extracellular Ca^{2+}, suggesting that cyclic AMP and PKC activators sensitize the secretory apparatus to Ca^{2+} (Hughes *et al.*, 1987, 1989). In previous studies using permeabilized cells, cyclic AMP and PMA have been shown to increase Ca^{2+}-induced insulin secretion (Tamagawa *et al.*, 1985; Jones *et al.*, 1985, 1986, 1989; Wollheim *et al.*, 1987; Vallar *et al.*, 1987), again compatible with a sensitization mechanism. However, the ability of PMA and cyclic AMP to increase the maximal rate of Ca^{2+}-induced secretion (Jones *et al.*, 1989) suggests a component in addition to sensitization to Ca^{2+}. The present study provides further evidence for such a component of the insulin secretory process, since both cyclic AMP and PMA could stimulate insulin release from Ca^{2+}-insensitive islets.

Studies using permeabilized cells have produced few examples of Ca^{2+}-independent exocytosis (see Knight *et al.*, 1989). Attempts to totally remove intracellular Ca^{2+} by using high concentrations of chelators have been reported in permeabilized insulin-secreting cells and used as evidence for Ca^{2+}-independent secretion in response to activation of GTP-binding proteins (Vallar *et al.*, 1987; Wollheim *et al.*, 1987). In the present studies it is not possible to clearly define cyclic AMP- or PMA-induced secretion from Ca^{2+}-insensitive islets as Ca^{2+}-independent, since our incubation buffers always contained at least 50 nM-Ca^{2+}, which we find is essential to maintain islet integrity. Whether or not the effects seen in the present study are truly Ca^{2+}-independent, evidence is accumulating of a degree of independence in the secretory systems within B-cells. Thus secretory responses to Ca^{2+} are not dependent upon the activation of PKC, as shown in experiments using PKC-depleted islets (Hii *et al.*, 1987), nor upon the activation of PKA, as shown by the use of a specific cyclic AMP antagonist (Persaud *et al.*, 1990). Conversely, the present study provides evidence that an ability of the secretory system to respond to Ca^{2+} is not required for the B-cells to mount a secretory response to activators of PKA or PKC.

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