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Effects of protein kinase C activation on the regulation of the stimulus–secretion coupling in pancreatic β-cells

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Effects of protein kinase C (PKC) activation on the insulin-secretory process were investigated, by using β-cell-rich suspensions obtained from pancreatic islets of obese–hyperglycaemic mice. The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which is known to activate PKC directly, the muscarinic-receptor agonist carbamoylcholine and high glucose concentration enhanced the phosphorylation of a specific 80 kDa PKC substrate in the β-cells. At a non-stimulatory glucose concentration, 10 nM-TPA increased insulin release, although there were no changes in either the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) or membrane potential, as measured with the fluorescent indicators quin-2 and bisoxonol respectively. At a stimulatory glucose concentration TPA caused a lowering in $[Ca^{2+}]_i$, whereas membrane potential was unaffected. Despite the decrease in $[Ca^{2+}]_i$, there was a large stimulation of insulin release. Addition of TPA lowered $[Ca^{2+}]_i$ also in β-cells stimulated by tolbutamide or high K^+, although to a lesser extent than in those stimulated by glucose. There was no effect of TPA on either Ca^{2+} buffering or the ability of Ins(1,4,5)P_3 to release Ca^{2+} in permeabilized β-cells. However, the phorbol ester inhibited the rise in $[Ca^{2+}]_i$ in response to carbamoylcholine, which stimulates the formation of InsP_3, in intact β-cells. Down-regulation of PKC influenced neither glucose-induced insulin release nor the increase in $[Ca^{2+}]_i$. Hence, although PKC activation is of no major importance in glucose-stimulated insulin release, this enzyme can serve as a modulator of the glucose-induced insulin-secretory response. Such a modulation involves mechanisms promoting both amplification of the secretory response and lowering of $[Ca^{2+}]_i$.

INTRODUCTION

Insulin release from pancreatic β-cells is regulated by a variety of intracellular processes, including changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular levels of cyclic nucleotides and phosphoinositide turnover. The latter process is mediated by the activation of phospholipase C and results in generation of Ins(1,4,5)P_3 (hereafter InsP_3) and diacylglycerol (DAG) (for a review, see [1]). Whereas InsP_3 releases intracellularly bound Ca^{2+} [2], DAG exerts its effects through the activation of protein kinase C (PKC) (for a review, see [3]). Activity of this enzyme has been established in both rat [4] and mouse [5] pancreatic islets as well as insulin-producing tumour cells [6,7]. Nanomolar concentrations of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) are assumed to stimulate PKC specifically, and have been used extensively as a substitute for DAG [3].

Since the original observations that TPA stimulates insulin release [8,9], a number of investigations have indicated a role for PKC in insulin secretion. It has, for example, been demonstrated that TPA decreases the requirement of the secretory machinery for extracellular Ca^{2+} [10]. Furthermore, in permeabilized islets the phorbol ester increases insulin release at fixed sub-micromolar concentrations of Ca^{2+} [11,12]. The physiological response of the β-cell to various receptor agonists, such as the muscarinic-receptor agonist carbamoylcholine, implies an activation of phospholipase C (for a review, see [13]) and thus stimulation of PKC. It has been demonstrated that a high glucose concentration can activate phospholipase C and thereby stimulate the formation of InsP_3 and DAG in rat islets [14]. Further support for the notion that glucose activates PKC was given by the finding that stimulation with the sugar led to the phosphorylation of a 40 kDa protein, which was also phosphorylated by TPA [15,16]. However, it is still a matter of debate whether PKC has any major regulatory function in glucose-induced insulin release [17]. In the present study we have addressed not only the question of a possible role for PKC in glucose-stimulated insulin release, but also more specifically what steps are affected in the insulin-secretory mechanism subsequent to PKC activation. For this purpose we have studied pancreatic β-cells from obese–hyperglycaemic mice and mainly used TPA as an activator of PKC. In addition to insulin release, we have also measured changes in membrane potential and $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, and redistilled deionized water was used. Quin-2 acetoxymethyl ester (quin-2/AM), dimethyl sulphoxide (DMSO), mezerein and TPA were from Sigma, St. Louis, MO, U.S.A., and bisoxonol was from Molecular Probes, Junction City, OR, U.S.A. Diazoxide and crystalline rat insulin were

Abbreviations used: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; DMSO, dimethyl sulphoxide; DAG, diacylglycerol.

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supplied by Schering Corp., Kenilworth, NJ, U.S.A., and Novo A/S, Copenhagen, Denmark, respectively. Collagenase and Bio-Gel P-4 polyacrylamide beads were from Boehringer, Mannheim, Germany, and Bio-Rad Laboratories, Richmond, CA, U.S.A., respectively. Tolbutamide was a gift from Farbwerke Hoechst, Frankfurt, Germany, and D-600 was given by Knoll A.G., Ludwigshafen am Rhein, Germany.

Animals and preparation of cells

Adult obese–hyperglycaemic mice (*ob/ob*) of both sexes were taken from a local non-inbred colony [18] and starved overnight. The islets were isolated by a collagenase technique, and cell suspensions were prepared and cultured overnight, as previously described [19,20].

Media

The medium used for isolation of cells, measurements of $[Ca^{2+}]_i$, membrane potential and insulin release was a Hepes buffer, pH 7.4, containing 1.28 mM-Ca^{2+}, unless otherwise stated, and with Cl^- as the sole anion [21]. In all experiments, except for determinations of membrane potential, the medium was supplemented with 1 mg of bovine serum albumin/ml. For the studies of permeabilized cells a Hepes buffer, pH 7.0 (adjusted with KOH), containing 110 mM-KCl, 10 mM-NaCl, 2 mM-KH_2PO_4, 1 mM-$MgCl_2$ and 0.5 mg of bovine serum albumin/ml was used.

Immunoprecipitation of ^{32}P-labelled β-cell protein

The cells were labelled with ^{32}P (1 mCi/ml) for 3 h in a Hepes-buffered Krebs–Ringer bicarbonate salt solution (KRBH) (0.01 mM-phosphate) containing 17 mM-glucose and 1% fetal-calf serum at 37 °C in 5% CO_2 in air. Thereafter the cells were transferred to a similar solution containing only 5 mM-glucose and no ^{32}P. After a 30 min chase period, the cells were incubated with the respective test solutions for 10 min. The incubations were terminated by washing in ice-cold KRBH and lysis in a buffer containing 100 μl of 150 mM-NaCl, 50 mM-Tris, pH 7.4, 0.5% Nonidet P-40, 0.1% SDS, 5 mM-EDTA, 0.02% NaN_3, 100 k-units of Trasylol/ml and 10 mM-benzamidine. Nuclei were pelleted and the supernatants pre-cleaned with 20 μl of 50% (v/v) Protein A–Sepharose in the above lysis buffer, with the further addition of 2.5% bovine serum albumin. After pelleting of the Sepharose, the supernatant was immunoprecipitated as in [22] with an antibody against a 80 kDa PKC substrate [22]. The immunoprecipitated products were then separated by two-dimensional gel electrophoresis as previously described [23] before autoradiography. The results shown are representative of at least three separate experiments.

Measurement of PKC activity

PKC activity was measured as phosphorylation of histone III-S in the presence of 0.5 mM-$CaCl_2$, 100 μg of phosphatidylserine/ml and 200 nM-TPA, and given as arbitrary units of ^{32}P incorporated into histone III-S/μg of cellular DNA. The cells were homogenized [24] and the nuclei pelleted by centrifugation for 8 s at 12000 *g*. The pellets were used for DNA determination. The supernatant was centrifuged for 15 min at 150000 *g*, and the two fractions were then assayed for PKC activity [24]. The phosphorylated histones were separated by SDS/polyacrylamide-gel electrophoresis and quantified by densitometry.

Measurements of $[Ca^{2+}]_i$ and membrane potential

Islet cells were incubated for 45 min with 5 μM-quin-2/AM, resulting in a loading of about 1–3 nmol of quin-2/10^6 cells [25]. $[Ca^{2+}]_i$ was measured and calibrated as previously described [25]. Neither DMSO nor TPA affected cellular autofluorescence, as checked in separate experiments using cells not loaded with quin-2 (results not shown). The fluorescent dye bisoxonol [26] was used for estimations of qualitative changes in membrane potential in accordance with ref. [25]. All traces shown are typical of experiments repeated with at least three different cell preparations.

Measurements of insulin release

The dynamics of insulin release were studied by perifusing $(0.5–1) \times 10^6$ islet cells mixed with Bio-Gel P-4 polyacrylamide beads in a 0.5 ml column at 37 °C [27]. The flow rate was 0.3 ml/min, and 1–2 min fractions were collected and analysed for insulin radioimmunologically, with crystalline rat insulin as the standard.

Cell permeabilization and measurements of the ambient Ca^{2+} concentration

After being washed twice in cold permeabilization buffer, the cell suspension (0.3 ml) was permeabilized by exposure to high-voltage discharges (five pulses of 2.5 kV/cm). After permeabilization, the cells were centrifuged and the pellet was suspended in 25 μl of an identical buffer supplemented with 2 mM-MgATP and an ATP-regenerating system consisting of 10 mM-phosphocreatine and 20 units of creatine kinase/ml. The mitochondrial Ca^{2+} transport was blocked by 0.2 μM-antimycin and 1 μg of oligomycin/ml. The measurements were performed at room temperature as described previously, by using a Ca^{2+}-selective mini-electrode [28]. The recordings shown are typical for experiments performed with at least three different cell preparations.

Additions of TPA

Since it was observed that 0.1% ethanol had a depolarizing effect of its own on the β-cells, DMSO was chosen as the vehicle for diluting TPA and mezerein (Sigma) to 500-fold concentrated stock solutions. DMSO alone (final concn. 0.2%) was added in the control experiments. However, in the absence of glucose 0.2% DMSO interfered with the $[Ca^{2+}]_i$ increase induced by high K^+ (results not shown), but it did not affect the increase induced by 20 mM-glucose (Fig. 2*c*). To avoid this adverse effect, a 500-fold concentrated stock solution of TPA in DMSO/buffer (1:9, v/v) was used in experiments with low glucose concentrations.

RESULTS

Phosphorylation of an endogenous PKC substrate

To investigate phosphorylation induced specifically by PKC activation, an antibody directed against a 80 kDa PKC substrate [22] was used for immunoprecipitation and subsequent detection of phosphorylation *in situ* [22]. Fig. 1 shows the result of such an experiment. A marked increase in the accumulation of ^{32}P was observed with 100 μM-carbamoylcholine (Fig. 1*a*) or 100 nM-TPA (Fig. 1*b*), in the presence of 4 mM-glucose. A low level of

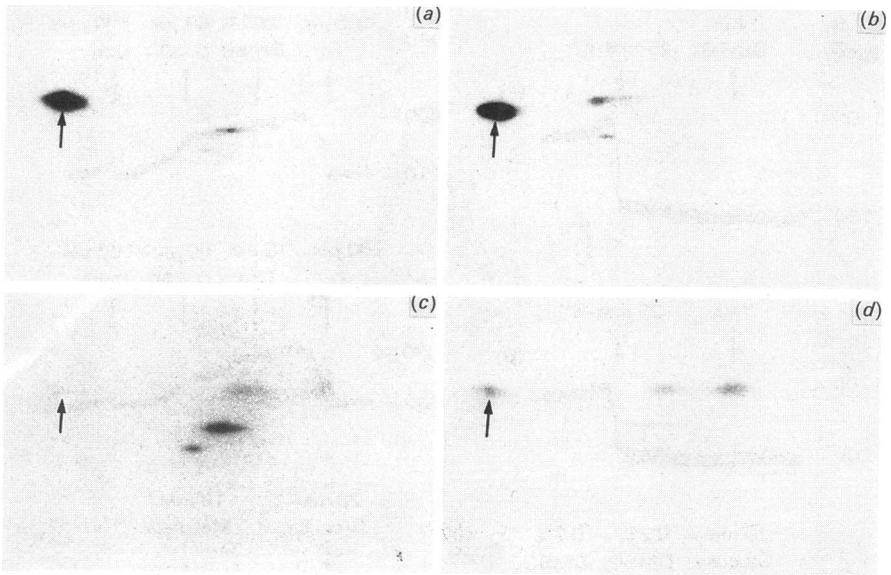

Fig. 1. Effects of 100 μM-carbamoylcholine (*a*), 100 nM-TPA (*b*) and 20 mM-glucose (*d*) on the phosphorylation of a 80 kDa PKC-specific substrate protein imunoprecipitated with a monoclonal antibody (arrow)

In (*a*) and (*b*), 4 mM-glucose was present during the incubations. Autoradiographs of two-dimensional SDS/polyacrylamide gels from the same batch of cells are shown. For comparison, (*c*) shows the phosphorylation in the presence of 4 mM-glucose.

phosphorylation was detected in the presence of a non-stimulatory (4 mM) glucose concentration (Fig. 1*c*). Interestingly, when the concentration of the sugar was increased to 20 mM (Fig. 1*d*), the accumulation of ^{32}P in the 80 kDa substrate increased. Hence, our data suggest that not only carbamoylcholine and TPA, but also glucose, phosphorylate a specific 80 kDa PKC substrate protein.

Effects of PKC activation on $[Ca^{2+}]_i$

Fig. 2 outlines the effects of TPA on $[Ca^{2+}]_i$ in quin-2-loaded islet cells. In the presence of 4 mM-glucose, i.e. at resting $[Ca^{2+}]_i$, addition of 10 nM-TPA had no detectable effect (Figs. 2*a* and 2*b*). Under these conditions, there was no effect of TPA on the subsequent K^+-induced rise in $[Ca^{2+}]_i$. However, when the β-cells were depolarized by 20 mM-glucose (Figs. 2*c* and 2*d*), 10 nM-TPA induced a pronounced decrease in $[Ca^{2+}]_i$, no further effect being observed on increasing the concentration of TPA to 100 nM. In Figs. 2(*e*) and 2(*f*) $[Ca^{2+}]_i$ was raised by a combination of 4 mM-glucose and 100 μM-tolbutamide. In this case 10 nM-TPA also decreased $[Ca^{2+}]_i$, but to a lesser extent than in the presence of 20 mM-glucose. Control additions of DMSO alone did not affect $[Ca^{2+}]_i$ (Figs. 2*a*, 2*c* and 2*e*). In Figs. 2(*e*) and 2(*f*) 50 μM-D-600 was added to block the voltage-activated Ca^{2+} channels. When $[Ca^{2+}]_i$ had returned to the basal level, the cells were stimulated with 100 μM-carbamoylcholine to induce $InsP_3$ formation, with subsequent release of intracellular Ca^{2+} [14,28]. In the presence of 10 nM-TPA the resulting Ca^{2+} peak was smaller (compare Figs. 2*e* and 2*f*). The effects of TPA on glucose-depolarized β-cells were reproduced by using 10 nM-mezerein, another activator of PKC [29] that is structurally different from the phorbol ester (Fig. 2*g*). Mezerein is less tumorigenic and has a lower PKC binding affinity than TPA [30]. In order to down-regulate PKC, islet cells were pretreated with 200 nM-TPA for 24 h. As is obvious from Fig. 3, such a pretreatment did not affect the glucose-induced increase in $[Ca^{2+}]_i$. However, in these cells 10 nM-TPA failed to decrease $[Ca^{2+}]_i$ (Fig. 3*b*), an effect obtained by the addition of D-600. Acute stimulation with TPA also failed to suppress the carbamoylcholine-induced increase in $[Ca^{2+}]_i$ in phorbol-ester-treated cells (Fig. 3*b*).

Since the experimental protocol used in Figs. 2(*a*) and 2(*b*) may not permit the detection of small effects on the depolarization-induced rise in $[Ca^{2+}]_i$ in response to an increase in K^+, another approach was tested (Fig. 4). Cells were depolarized with 25 mM-K^+ in the absence of glucose (Figs. 4*a* and 4*b*), in the presence of 4 mM-glucose (Figs. 4*c* and 4*d*) or at 20 mM-glucose (Figs. 4*e* and 4*f*). To prevent depolarization at the highest glucose concentration, 400 μM-diazoxide was added [25]. It could thus be demonstrated that addition of 10 nM-TPA increased the rate of decline in $[Ca^{2+}]_i$, by approx. 200–300 nM during 6 min, irrespective of the glucose concentration. However, the relative decline in $[Ca^{2+}]_i$, compared with the extrapolated value expected in the absence of TPA (broken lines drawn by hand), was more pronounced at 20 mM-glucose ($51 \pm 2\%$, $n = 4$) than at 4 mM ($40 \pm 2\%$, $n = 3$) or at 0 mM ($28 \pm 7\%$, $n = 3$) of the sugar ($P < 0.01$ and $P < 0.05$ respectively versus 20 mM-glucose). Values are given as means ± S.E.M. for the indicated numbers of experiments, and statistical significances were calculated by Student's *t* test. These findings are probably accounted for by a more pronounced Ca^{2+} buffering at higher concentrations of glucose.

Effects of PKC activation on outward transport and intracellular uptake of Ca^{2+}

It has been reported that activation of PKC with TPA can attenuate or delay increases in $[Ca^{2+}]_i$ promoted by formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) [31], an abrupt increase in the external Ca^{2+} concentration [32], treatment with the Ca^{2+} ionophore A23187 [32] and depolarization with high K^+ [33,34]. The effects have

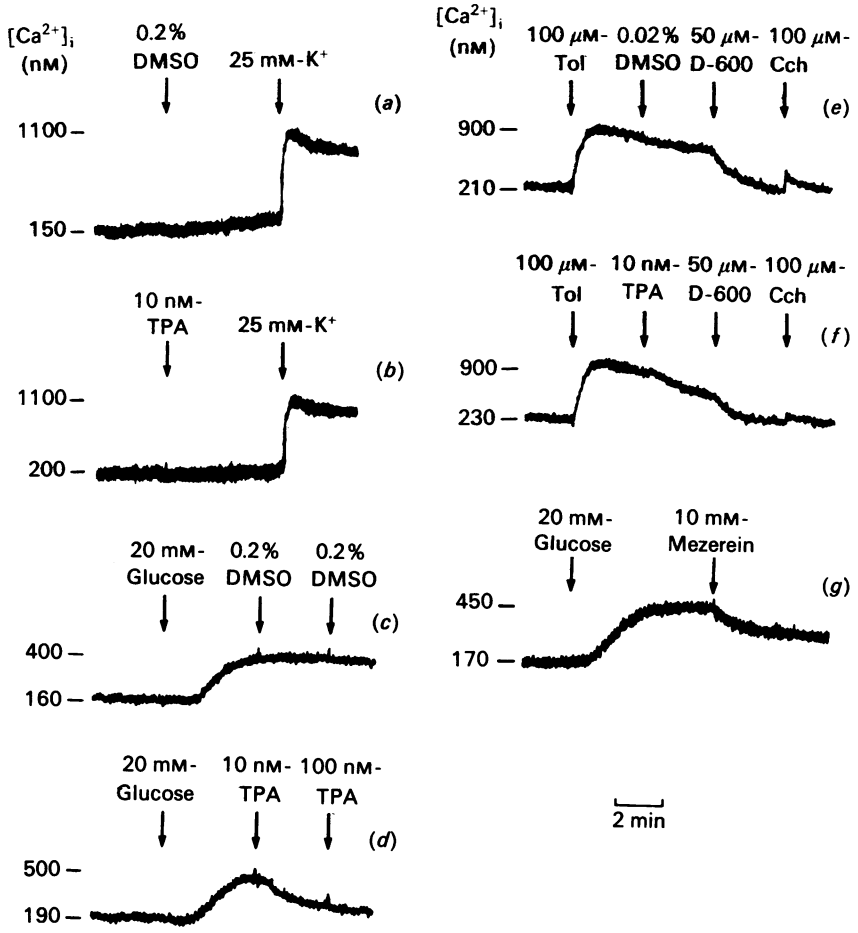

Fig. 2. Effects of DMSO, TPA, glucose, tolbutamide (Tol), D-600, carbamoylcholine (Cch) and mezerein on $[Ca^{2+}]_i$ of islet-cell suspensions

In (*a*), (*b*), (*e*) and (*f*) 4 mM-glucose was present throughout the experiments.

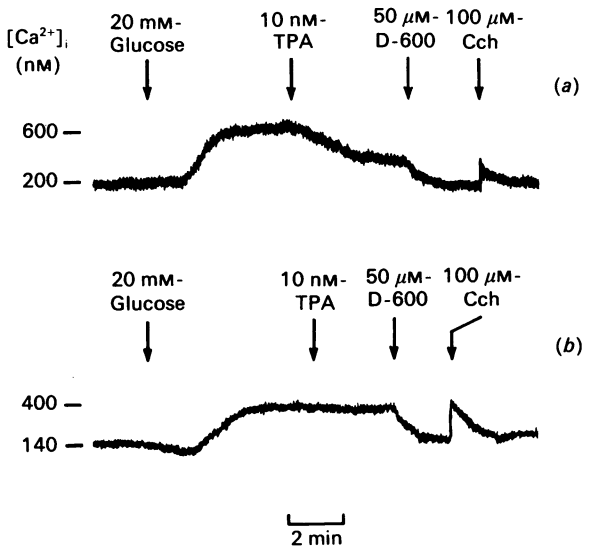

Fig. 3. Effects of glucose, TPA, D-600 and carbamoylcholine (Cch) on $[Ca^{2+}]_i$ in islet-cell suspensions cultured for 24 h in the presence of 200 nM-TPA (*b*)

Control cells (*a*) were cultured for the same time period in the presence of 0.1% DMSO.

been attributed both to PKC stimulation of outward Ca^{2+} transport [31–33] and to PKC-mediated blockage of voltage-activated Ca^{2+} channels in excitable cells [34]. Either of these mechanisms or both might be responsible for the effects observed in the β-cells. With the experiments shown in Fig. 5, an attempt was made to clarify whether PKC activation indeed stimulates Ca^{2+} efflux in pancreatic β-cells. A rise in $[Ca^{2+}]_i$ was induced by increasing the Ca^{2+} concentration of an initially Ca^{2+}-deficient medium, from about 10 μM to 2 mM. To exclude Ca^{2+} entry through the voltage-activated Ca^{2+} channels, D-600 was added. The initial experiments were performed in the presence of 20 mM-glucose (Figs. 5*a* and 5*b*), since the effects of TPA on $[Ca^{2+}]_i$ were most evident at this concentration (cf. Figs. 2d and 4*f*). In β-cells treated with 10 nM-TPA, the dynamics of the $[Ca^{2+}]_i$ rise showed no tendency towards delay, and the final $[Ca^{2+}]_i$ values were not different from those of control cells (Figs. 5*a* and 5*b*). Since the presence of 20 mM-glucose might obscure a possible action of TPA, owing to an increased Ca^{2+}-buffering capacity of the β-cells [35], experiments were also performed in the absence of the sugar (Figs. 5*c* and 5*d*). In this case, K^+ was added to verify that the voltage-activated Ca^{2+} channels had been blocked by the addition of D-600 (not shown in Fig. 5). Under these conditions, TPA had no detectable effects. It should be mentioned

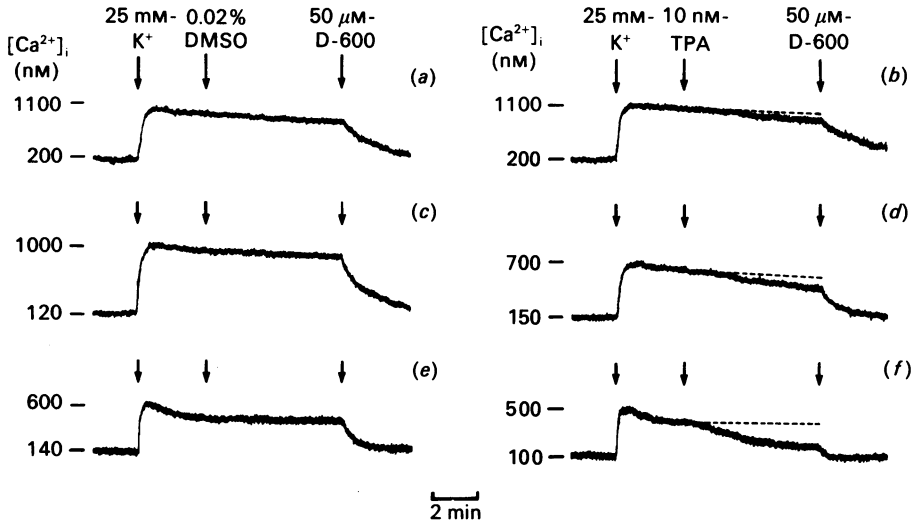

Fig. 4. Effects of DMSO and TPA on the K^+-induced increase in $[Ca^{2+}]_i$ of islet-cell suspensions in the absence of glucose (*a* and *b*), in the presence of 4 mM-glucose (*c* and *d*), or in the presence of 20 mM-glucose and 400 μM-diazoxide (*e* and *f*)

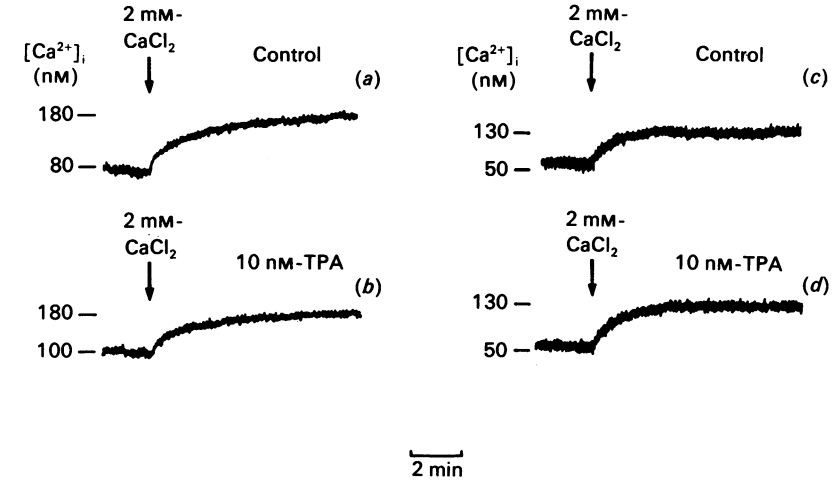

Fig. 5. Effects of DMSO and TPA on the $[Ca^{2+}]_i$ rise in islet-cell suspensions after addition of 2 mM-Ca^{2+} to a Ca^{2+}-deficient medium, in the presence of 20 mM-glucose and 50 μM-D-600 (*a* and *b*) or 25 mM-K^+ and 50 μM-D-600 (*c* and *d*)

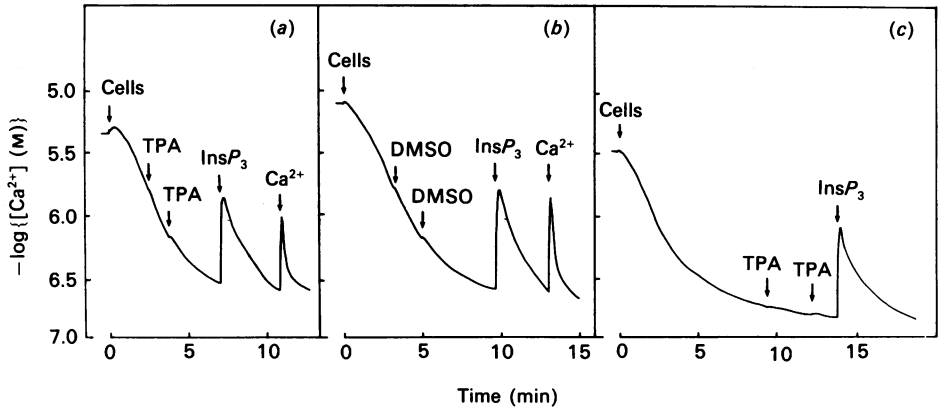

Fig. 6. Effects of DMSO (0.1%), TPA (successive additions of 10 and 100 nM), Ca^{2+} (0.125 nmol) and $InsP_3$ (6 μM) on the ambient Ca^{2+} concentration maintained by permeabilized pancreatic β-cells

The additions of cells as well as the various test substances are indicated by the arrows.

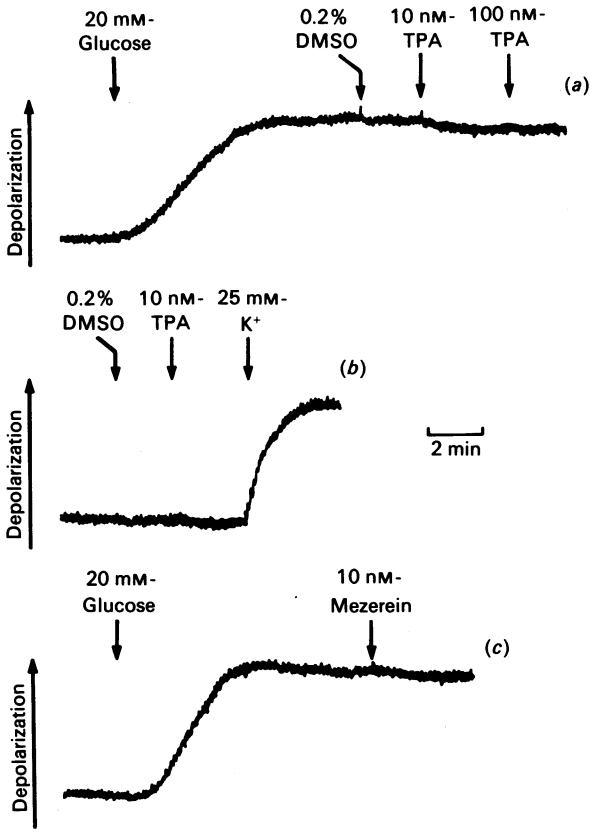

Fig. 7. Effects of glucose, K^+, DMSO, TPA and mezerein on membrane potential of islet-cell suspensions as measured with bisoxonol

In (*b*) 4 mM-glucose was present from the beginning of the experiment.

that the quin-2 loading values for TPA-treated as well as control cells were similar, and the experiments were paired for the sake of comparison.

To establish a possible effect of PKC activation on the intracellular Ca^{2+} metabolism, permeabilized normal pancreatic β-cells were investigated (Fig. 6). In such experiments, addition of TPA failed to affect the buffering rate of Ca^{2+} (Figs. 6*a* and 6*c*). Furthermore, there was no effect of the phorbol ester on the subsequent release of Ca^{2+} induced by $InsP_3$ (Fig. 6*c*). In the experiments with the permeabilized cells, addition of DMSO alone served as a control (Fig. 6*b*). Experiments were also performed with permeabilized cells incubated with TPA for 15–30 min before permeabilization, revealing similar effects of the phorbol ester to those presented in Fig. 6 (results not shown). These experiments were done to ensure that the lack of acute effects of TPA was not due to loss of cytosolic PKC activity during permeabilization.

Effects of PKC activation on membrane potential

In search for a possible mechanism behind the lowering effect of TPA on $[Ca^{2+}]_i$, changes in membrane potential were assessed by using bisoxonol. As shown in Fig. 7, glucose and K^+ depolarized the cell suspensions. In these experiments TPA had no effect under basal conditions (Fig. 7*b*). In the presence of 20 mM-glucose neither the phorbol ester nor mezerein evoked any major effects (Figs. 7*a* and 7*c*). The small decline in fluorescence induced by TPA was probably accounted for by the lipophilicity of this compound [30]. This property might then lead to a slight shift in the bisoxonol distribution in the cell membrane. It should be remembered that these measurements do not exclude the existence of small changes in the intricate electrical response pattern of

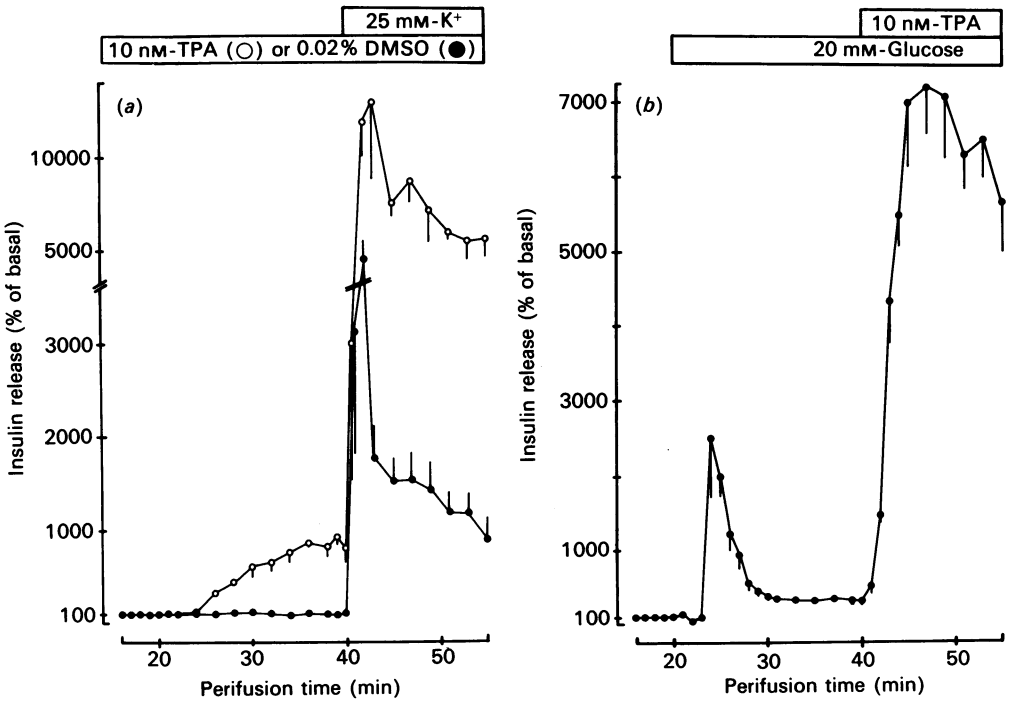

Fig. 8. Effects of glucose, TPA, DMSO and K^+ on the dynamics of insulin release from column-perifused islet-cell suspensions

In (*a*), 4 mM-glucose was present throughout the perifusions, and in the control perifusions (*a*, lower trace) 0.2% DMSO was introduced instead of TPA. The average insulin release during the first 5 min was taken as 100%, and subsequent values are given relative to this value. Mean values ± S.E.M. for three or four experiments are shown.

glucose-stimulated β-cells. Nevertheless, the present data do not support repolarization being responsible for the lowering effect of TPA on $[Ca^{2+}]_i$.

Effects of PKC activation on insulin release

In order to correlate changes in $[Ca^{2+}]_i$ with changes in insulin release, the latter parameter was studied in a column perifusion system (Fig. 8). At 4 mM-glucose, exposure to 10 nM-TPA induced a gradual but marked increase in insulin release, and a plateau was reached after about 10 min of perifusion (Fig. 8*a*). It should be noted that no increase in $[Ca^{2+}]_i$ was detected under similar conditions (cf. Fig. 2*b*). At the subsequent depolarization with 25 mM-K^+, both the control cells and the TPA-stimulated cells responded with a pronounced increase in insulin release, although the maximal release was larger in the latter case. Fig. 8(*b*) shows that 20 mM-glucose produced an initial peak in insulin release, followed by the establishment of a new steady-state level. The introduction of 10 nM-TPA caused a dramatic further increase in secretion, despite the concomitant decrease in $[Ca^{2+}]_i$ (cf. Fig. 2*d*). In cells pretreated with 200 nM-TPA for 24 h, the stimulatory effect of glucose on insulin release was not significantly different from that in control cells (Fig. 9). However, in the former cells, subsequent stimulation with 10 nM of the phorbol ester failed to promote an increase in insulin release. When the activity of PKC was assessed after 24 h treatment with 200 nM-TPA, the phosphorylation of histone III-S, as determined by densitometry, was decreased to $16 \pm 4\%$ (mean $\pm$ S.E.M. for three experiments) of control ($P < 0.01$, by Student's t test for paired data), demonstrating that down-regulation of the enzyme actually had taken place. Although not so accentuated, there was a tendency to a redistribution of the subcellular localization of PKC activity after treatment with 200 nM-TPA for 24 h. The percentage of PKC activity in the supernatant was 59 ± 9.6 in the control group and 43 ± 7.5 in the group pretreated with 200 nM-TPA. These values are means $\pm$ S.E.M. for three experiments.

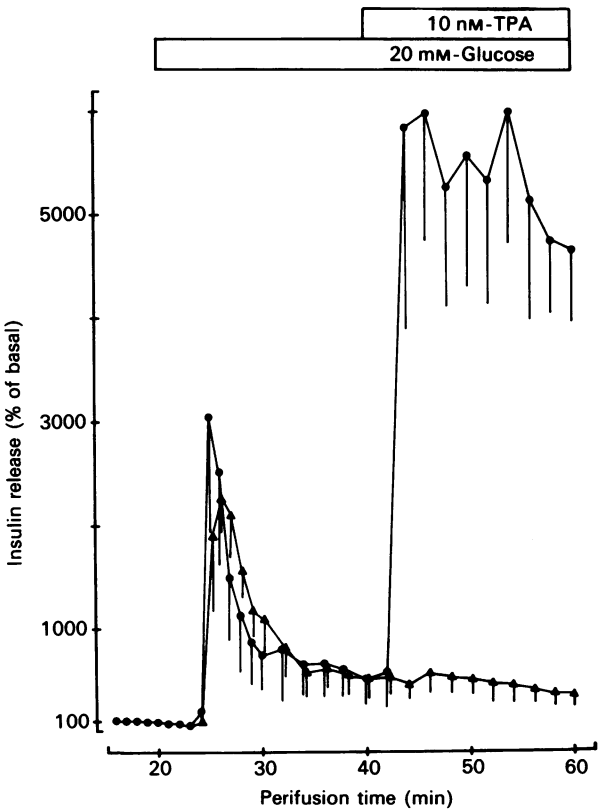

Fig. 9. Effects of glucose and TPA on the dynamics of insulin release from column-perifused islet-cell suspensions

The cell suspensions were cultured for 24 h in the presence of 200 mM-TPA (▲) or 0.1% DMSO (●) before the perifusions. Mean values $\pm$ S.E.M. for three experiments are shown.

DISCUSSION

By immunoprecipitation of a specific 80 kDa PKC-substrate protein of islet cells labelled with ^{32}P, the present study revealed an increased PKC activity in response not only to TPA and carbamoylcholine but also to glucose. Glucose stimulation of PKC activity has previously been suggested in rat islets [15], but to our knowledge this is the first study indicating that the sugar indeed induces the phosphorylation of a clearly defined PKC substrate in mouse pancreatic β-cells. It has previously been shown that glucose stimulation leads to an increased DAG production [36] and that the generation of DAG probably involves an activation of phospholipase C [14], as well as synthesis *de novo* through glycolytic intermediates [36]. It is, however, possible that the effect of glucose on phospholipase C results, at least in part, from the depolarization-induced increase in $[Ca^{2+}]_i$ [37], which in turn results in an activation of the enzyme [14].

It is noteworthy that PKC activation induces a lowering in $[Ca^{2+}]_i$. When discussing possible mechanism(s) for such an effect, a previous study suggested a direct block of the voltage-activated Ca^{2+} channels in RINm5F cells [34]. However, this was not verified from direct electrophysiological measurements, but was rather inferred from the observation that PKC activation resulted in a lowering of $[Ca^{2+}]_i$. On the contrary, by using the whole-cell mode of the patch-clamp technique we previously demonstrated that 10 nM-TPA induced an increase in the inward Ca^{2+} currents [38], arguing against a direct interference with the voltage-activated Ca^{2+} channels being a contributing factor. In this context, it should be emphasized that TPA also failed to affect either glucose- or tolbutamide-stimulated $^{45}Ca^{2+}$ uptake in normal β-cells [9,39]. In addition, TPA has been found to increase $^{45}Ca^{2+}$ efflux, in both mouse and rat pancreatic islets, under a variety of experimental conditions, such as in the absence of extracellular Ca^{2+} [9] and in the presence of tolbutamide [39]. As suggested from both the present and previous studies [9,39], demonstrating no effect of TPA on $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ under basal conditions, it is not likely that the lowering effect of the phorbol ester on $[Ca^{2+}]_i$ is due to an interference with unspecific leakage of Ca^{2+}. Since TPA was without effect on Ca^{2+} buffering in permeabilized β-cells, it is most likely accounted for by an active extrusion of Ca^{2+} from the cell. A plausible candidate responsible for the extrusion is the plasma-membrane ATP-dependent Ca^{2+} pump. This enzyme has indeed been found to be stimulated by TPA in plasma-membrane-enriched fractions from neutrophils [31]. In those cells, such a stimulation prevented the rise in $[Ca^{2+}]_i$ normally obtained in response to the chemotactic

peptide fMet-Leu-Phe. It cannot be excluded that part of the lowering effect of TPA is due to a direct interference with the formation of InsP_3 and thereby a decreased mobilization of intracellularly bound Ca^{2+} [40,41]. That PKC activation might inhibit the formation of InsP_3 in β-cells, a phenomenon previously described for RINm5F cells [42] as well as other cells [40,41], was supported by the finding that carbamoylcholine was less effective in raising $[Ca^{2+}]_i$ after addition of TPA. In this context it should be noted that previous studies in permeabilized RINm5F cells [33] have failed to demonstrate an interference of the phorbol ester with the InsP_3-induced Ca^{2+} release itself, a finding that was now corroborated in permeabilized mouse pancreatic β-cells.

When considering effects of TPA on different ionic channels, Wollheim and co-workers have recently proposed that activators of PKC depolarize the RINm5F cells by closing the ATP-regulated K^+ channels [43]. However, this is probably not the case in β-cells, since TPA failed to induce an increase in membrane potential (the present study, and [39]) under conditions where the RINm5F cells responded with a slight depolarization [33]. In addition, no effects of TPA were observed on the $^{86}Rb^+$ efflux (used as a marker for K^+ efflux) from isolated rat and mouse islets [9,39].

The identification of PKC in intact islet cells [4,5] and the fact that the intracellular concentration of diacylglycerol is elevated during stimulation of insulin release by glucose [13,15,36] have led to the suggestion that this enzyme is involved in the regulation of the stimulus–secretion coupling in pancreatic β-cells. Interestingly, Zawalich *et al.* have demonstrated that a combination of phorbol ester with a Ca^{2+} ionophore produced a biphasic secretory response, which is similar to that after glucose stimulation [44]. Further support for a role of PKC in the regulation of insulin release was given by the finding that TPA-induced potentiation of secretion was inhibited by the PKC inhibitors clomiphene [45] and H-7 [17]. In contradistinction to these findings, Hii and co-workers claimed to have depleted pancreatic islets of PKC activity and still observed insulin release in response to glucose, suggesting to them that PKC may not play an essential role in the regulation of glucose-induced insulin release [46]. The latter findings are strengthened by our results, demonstrating that the effect of glucose, not only on insulin release but also on $[Ca^{2+}]_i$, persisted despite down-regulation of PKC. Whatever the physiological role of PKC activation might be in the pancreatic β-cell, there is no doubt that stimulation with phorbol esters leads to a marked increase in insulin release, as is evident from the present study. The fact that we obtained more pronounced effects of TPA on insulin release than have previously been reported in isolated rat and mouse islets [8–10,39] might be explained by a higher sensitivity of the secretory machinery to TPA in β-cells obtained from the *ob/ob* mouse.

From studies on permeabilized chromaffin cells, it has been suggested not only that the mechanism responsible for the stimulatory effect of PKC on secretion is accounted for by an increased sensitivity of the secretory machinery to $[Ca^{2+}]_i$, but also that this enzyme is intimately involved in the control machinery of exocytosis [47,48]. As demonstrated in the present study, PKC activation in normal pancreatic β-cells leads to a complicated response pattern. Under basal conditions the increased insulin release is not paralleled by a change in $[Ca^{2+}]_i$, which is in agreement with what has been found in other secretory cells [49]. On the other hand, at high glucose concentrations PKC activation further enhances insulin release, but in this case $[Ca^{2+}]_i$ is decreased. Although glucose is able to promote PKC activation, the down-regulation experiments clearly demonstrate that this enzyme is of no major significance in glucose-stimulated insulin release. Hence the role of PKC in the insulin secretory process is most likely of a modulatory nature. In addition to serving as an amplifying signal for the release of the hormone [10,12], PKC activation might function as a negative-feedback modulator of stimulated Ca^{2+} influx and thereby assist in recovery from raised $[Ca^{2+}]_i$, as previously suggested in other cell types [32,50,51].

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REFERENCES

1. Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
2. Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) Nature (London) **306**, 67–69
3. Nishizuka, Y. (1984) Nature (London) **308**, 693–698
4. Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y. & Nishizuka, Y. (1982) FEBS Lett. **138**, 183–186
5. Thams, P., Capito, K. & Hedeskov, C. J. (1984) Biochem. J. **221**, 247–253
6. Lord, J. M. & Ashcroft, S. J. H. (1984) Biochem. J. **219**, 547–551
7. Hutton, J. C., Peshavaria, M. & Brocklehurst, K. W. (1984) Biochem. J. **224**, 483–490
8. Virji, M. A. G., Steffes, M. W. & Estensen, R. D. (1978) Endocrinology (Baltimore) **102**, 706–711
9. Malaisse, W. J., Sener, A., Herchuelz, A., Carpinelli, A. R., Poloczek, P., Winand, J. & Castagna, M. (1980) Cancer Res. **40**, 3827–3831
10. Hughes, S. J., Christie, M. R. & Ashcroft, S. J. H. (1987) Mol. Cell. Endocrinol. **50**, 231–236
11. Tamagawa, T., Niki, H. & Niki, A. (1985) FEBS Lett. **183**, 430–432
12. Jones, P. M., Stutchfield, J. & Howell, S. L. (1985) FEBS Lett. **191**, 102–106
13. Best, L. & Malaisse, W. J. (1983) Diabetologia **25**, 299–305
14. Biden, T. J., Peter-Riesch, B., Schlegel, W. & Wollheim, C. B. (1987) J. Biol. Chem. **262**, 3567–3571
15. Dunlop, M. E. & Larkins, R. G. (1986) Arch. Biochem. Biophys. **248**, 562–569
16. Harrison, D. E., Ashcroft, S. J. H., Christie, M. R. & Lord, J. M. (1984) Experientia **40**, 1075–1084
17. Metz, S. A. (1988) Diabetes **37**, 3–7
18. Hellman, B. (1965) Ann. N.Y. Acad. Sci. **131**, 541–558
19. Lernmark, Å. (1974) Diabetologia **10**, 431–438
20. Arkhammar, P., Rorsman, P. & Berggren, P.-O. (1986) Biosci. Rep. **6**, 355–361

21. Hellman, B. (1975) Endocrinology (Baltimore) **97**, 392–398
22. Albert, K. A., Walaas, S. I., Wang, J. K.-T. & Greengard, P. C. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2822–2826
23. Perdew, G. H., Schaup, H. W. & Selivonchick, D. P. (1983) Anal. Biochem. **135**, 453–455
24. Brocklehurst, K. W. & Hutton, J. C. (1984) Biochem. J. **220**, 283–290
25. Arkhammar, P., Nilsson, T., Rorsman, P. & Berggren, P.-O. (1987) J. Biol. Chem. **262**, 5448–5454
26. Rink, T. J., Montecucco, C., Hesketh, T. R. & Tsien, R. Y. (1980) Biochim. Biophys. Acta **595**, 15–30
27. Kanatsuna, T., Lernmark, Å., Rubenstein, A. H. & Steiner, D. F. (1981) Diabetes **30**, 231–234
28. Nilsson, T., Arkhammar, P., Hallberg, A., Hellman, B. & Berggren, P.-O. (1987) Biochem. J. **248**, 329–336
29. Miyake, R., Tanaka, Y., Tsuda, T., Kaibuchi, K., Kikkawa, U. & Nishizuka, Y. (1984) Biochem. Biophys. Res. Commun. **121**, 649–656
30. Dell Aquila, M. L., Warren, B. S., de Vries, D. J. & Blumberg, P. M. (1987) in Development and Recognition of the Transformed Cell (Greene, M. I. & Hamaoka, T., eds.), pp. 157–185, Plenum Publishing Corp., New York
31. Lagast, H., Pozzan, T., Waldvogel, F. A. & Lew, P. D. (1984) J. Clin. Invest. **73**, 878–883
32. Richard, J. E. & Sheterline, P. (1985) Biochem. J. **231**, 623–628
33. Arkhammar, P., Nilsson, T. & Berggren, P.-O. (1986) Biochim. Biophys. Acta **887**, 236–241
34. Di Virgilio, F., Pozzan, T., Wollheim, C. B., Vicentini, L. M. & Meldolesi, J. (1986) J. Biol. Chem. **261**, 32–35
35. Hellman, B. & Gylfe, E. (1986) in Calcium and Cell Function (Cheung, W. Y., ed.), vol. 6, pp. 253–326, Academic Press, New York
36. Peter-Riesch, B., Fathi, M., Schlegel, W. & Wollheim, C. B. (1988) J. Clin. Invest. **81**, 1154–1161
37. Rorsman, P., Abrahamsson, H., Gylfe, E. & Hellman, B. (1984) FEBS Lett. **170**, 196–200
38. Rorsman, P., Arkhammar, P. & Berggren, P.-O. (1986) Am. J. Physiol. **251**, C912–C919
39. Henquin, J.-C., Bozem, M., Schmeer, W. & Nenquin, M. (1987) Biochem. J. **246**, 393–399
40. Orellana, S. A., Solski, P. A. & Heller Brown, J. (1985) J. Biol. Chem. **260**, 5236–5239
41. Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1986) FEBS Lett. **201**, 137–142
42. Biden, T. J., Vallar, L. & Wollheim, C. B. (1988) Biochem. J. **251**, 435–440
43. Wollheim, C. B., Dunne, M. J., Peter-Riesch, B., Bruzzone, R., Pozzan, T. & Petersen, O. H. (1988) EMBO J. **7**, 2443–2449
44. Zawalich, W., Brown, C. & Rasmussen, H. (1983) Biochem. Biophys. Res. Commun. **117**, 448–455
45. Hughes, S. J. & Ashcroft, S. J. H. (1988) Biochem. J. **249**, 825–830
46. Hii, C. S. T., Jones, P. M., Persaud, S. J. & Howell, S. L. (1987) Biochem. J. **246**, 489–493
47. Knight, D. E. & Baker, P. F. (1983) FEBS Lett. **160**, 98–100
48. Knight, D. E., Sugden, D. & Baker, P. F. (1988) J. Membr. Biol. **104**, 21–34
49. Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) Nature (London) **305**, 317–319
50. Drummond, A. H. (1985) Nature (London) **315**, 752–755
51. MacIntyre, D. E., McNicol, A. & Drummond, A. H. (1985) FEBS Lett. **180**, 160–164

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