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Activators of protein kinase C depolarize insulin-secreting cells by closing K^+ channels

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Carbohydrate stimuli of insulin secretion depolarize the pancreatic B cell and the B-cell line RINm5F by inhibiting ATP-sensitive K^+ channels. We examined the possibility that this effect is mediated by activation of protein kinase C. In RINm5F cells, the triose D-glyceraldehyde evoked a rapid increase of the mass of 1,2-diacylglycerol, the endogenous activator of protein kinase C. This effect is mainly due to *de novo* synthesis of the lipid from glycolytic intermediates, as glyceraldehyde carbon was incorporated into 1,2-diacylglycerol within 1 min of exposure to ^{14}C-labelled glyceraldehyde. The effects of two exogenous activators of kinase C, 4-β-12-phorbolmyristate 13-acetate (PMA) and 1,2-didecanoylglycerol (DC_{10}) on single K^+ channel currents were examined in RINm5F cell-attached membrane patches. Both PMA and DC_{10} depolarized the cells and decreased the open-state probability of the ATP-sensitive K^+ channels. These actions were not due to changes in cellular ATP content, since PMA, like glyceraldehyde, failed to alter cellular ATP. As is the case for glyceraldehyde, PMA and DC_{10} raised cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and stimulated insulin secretion. Both of these effects are inhibited in the absence of external Ca^{2+}. This, and the attenuation of the $[Ca^{2+}]_i$ rise by verapamil, suggest that all three stimuli raise $[Ca^{2+}]_i$ by promoting Ca^{2+} influx through voltage-gated channels in turn leading to insulin secretion. As the exogenous activators of protein kinase C mimic the effects of glyceraldehyde, it is proposed that the carbohydrate-mediated production of 1,2-diacylglycerol constitutes the link between metabolism and membrane depolarization. The critical initiating event in carbohydrate-stimulated insulin secretion may thus be the closure of K^+ channels due to the activation of protein kinase C.

Key words: K^+ channels/protein kinase C/phorbol esters/diacylglycerol

Introduction

The regulation of insulin secretion from the pancreatic B cells is the single most important factor governing blood glucose homeostasis and failure to recognize glucose as a secretagogue characterizes many pathophysiological states including diabetes mellitus (Lernmark, 1985). A rise in cytosolic Ca^{2+}, $[Ca^{2+}]_i$ is thought to precede and cause the stimulation of insulin release by carbohydrates (glucose and glyceraldehyde) and amino acids (e.g. alanine) (Wollheim *et al.*, 1984; Wollheim and Biden, 1986a; Arkhammer *et al.*, 1987). In turn the rise of $[Ca^{2+}]_i$ is primarily due to gated Ca^{2+} influx through voltage-dependent Ca^{2+} channels during membrane depolarization (Velasco *et al.*, 1988), although Ca^{2+} mobilization may also contribute to the rise (Wollheim and Biden, 1986a). Both glucose and glyceraldehyde depolarize insulin-secreting cells by inhibiting a particular type of K^+ channel (Arkhammer *et al.*, 1987; Ashcroft *et al.*, 1984; Dunne *et al.*, 1986; Misler *et al.*, 1986; Petersen and Findlay, 1987), which can also be inhibited by internal ATP (referred to as K^+ATP channel) (Cook and Hales, 1984; Findlay *et al.*, 1985b; Rorsman and Trube, 1985). It has therefore been suggested that changes in ATP (Ashcroft *et al.*, 1984; Dunne *et al.*, 1986; Misler *et al.*, 1986; Petersen and Findlay, 1987; Cook and Hales, 1984) or the ATP/ADP ratio (Misler *et al.*, 1986; Dunne and Petersen, 1986; Kakei *et al.*, 1986) could mediate the effect of carbohydrates. However, at present it is not clear whether ATP mediates any of the effects of nutrients on K^+ATP channels in insulin-secreting cells. In fact, in intact islet cells, glucose only raises ATP levels significantly after prolonged nutrient deprivation (Kakei *et al.*, 1986; Ashcroft *et al.*, 1973; Malaisse and Sener, 1987; Ashcroft *et al.*, 1987). Moreover, in the only study in which the early time points, relevant to the initial depolarization, were examined, an elevation in glucose from 1.7 to 16.7 mM caused a decrease in ATP concentration at 1 min, followed by a slow gradual increase (Malaisse *et al.*, 1979).

Therefore, alternative explanations for the coupling of carbohydrate metabolism to changes in membrane potential in insulin-secreting cells were examined. Several types of ion channel have been shown to be modulated by activation of the Ca^{2+} and phospholipid-dependent protein kinase C (Nishizuka, 1986; Kaczmarek, 1987). Thus, phorbol esters and synthetic diacylglycerols, which activate protein kinase C, have been reported to block K^+ currents (Kaczmarek, 1987; Higashida and Brown, 1986). It is unclear how the single channel currents are affected by protein kinase C.

The aim of this study was to examine (i) whether activation of protein kinase C alters the gating of K^+ATP assessed in cell-attached membrane patches of the insulin-secreting cell line RINm5F; (ii) whether the carbohydrate secretagogue glyceraldehyde could depolarize the cells by generating diacylglycerol, the endogenous activator of protein kinase C; and (iii) whether 12-phorbolmyristate 13-acetate (PMA) and 1,2-didecanoylglycerol (DC_{10}) exert similar effects on membrane potential, $[Ca^{2+}]_i$, and insulin secretion as glyceraldehyde. The results indicate that PMA and DC_{10} close the ATP-sensitive K^+ channels, depolarize the cells, raise $[Ca^{2+}]_i$ and stimulate insulin secretion. The involve-

ment of protein kinase C in glyceraldehyde-stimulated insulin secretion is further suggested by the finding that the triose increases the mass of cellular diacylglycerol by rapidly stimulating its *de novo* synthesis.

Results and discussion

Glyceraldehyde-induced 1,2-diacylglycerol production

Glyceraldehyde stimulates insulin secretion from RINm5F cells after a 2.5-min lag (Wollheim *et al.*, 1984). Therefore, total cellular 1,2-diacylglycerol was measured after either 2 or 5 min of incubation of the cells with carbohydrate (Table IA). A significant increase was observed at both time points, 70% of the response occurring already at 2 min. In normal pancreatic B cells, a similar time course has been seen for the formation of diacylglycerol following glucose stimulation (Peter-Riesch *et al.*, 1988). In contrast to receptor agonist-evoked diacylglycerol formation which is mainly due to phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (Wollheim and Biden, 1986a; Peter-Riesch *et al.*, 1988; Biden *et al.*, 1987), glucose promotes *de novo* synthesis of the lipid messenger in pancreatic islets (Peter-Riesch *et al.*, 1988; Dunlop and Larkins, 1985; Farese *et al.*, 1986; Vara and Tamarit-Rodriguez, 1986). Diacylglycerol is thus formed from its precursor phosphatidic acid (Dunlop and Larkins, 1985; Farese *et al.*, 1986) following the stepwise acylation of the glycolytic intermediates glycerol-3-phosphate and dihydroxyacetone phosphate via lysophosphatidic acid (Dunlop and Larkins, 1985). To test whether glyceraldehyde increases diacylglycerol levels in RINm5F cells by the same pathway, we exposed the cells acutely to ^{14}C-labelled glyceraldehyde. As can be seen in Table IB, radioactivity was incorporated into diacylglycerol within 1 min and increased further at 2 min. Thus, *de novo* synthesis of diacylglycerol could underlie the increase in cellular mass of the lipid messenger molecule. This effect was still observed when 2 mM EGTA was present during the pre-incubation and incubation (0.69 ± 0.02 pmol glyceraldehyde carbon incorporated per 10^6 cells/2 min, n = 4, cf. Table IB). This finding is similar to the situation observed with glucose in islets (Peter-Riesch *et al.*, 1988) and distinguishes diacylglycerol *de novo* synthesis from glucose-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis which is secondary to Ca^{2+} influx and can be completely abolished in the presence of EGTA (Wollheim and Biden, 1986a; Biden *et al.*, 1987).

Table I. Stimulation of 1,2-diacylglycerol generation by D-glyceraldehyde in RINm5F cells

Incubation time (min)	1	2	5
A.	1,2-diacylglycerol (ng/10^6 cells)		
Control	–	14.9 ± 0.7	15.3 ± 0.6
Glyceraldehyde (10 mM)	–	19.0 ± 0.9	21.6 ± 2.3
n		11	7
P		<0.005	<0.02
B.	Glyceraldehyde carbon incorporated (pmol/10^6 cells)		
Glyceraldehyde (1 mM)	0.33 ± 0.02	0.67 ± 0.06	–
n	8	8	–

A: effect on total mass; B: incorporation of ^{14}C from [^{14}C]glyceraldehyde into 1,2-diacylglycerol. RINm5F cells were incubated and 1,2-diacylglycerol measured as described in Materials and methods. The results represent mean ± SEM of 3–4 (A) and 2 (B) independent experiments. Statistical analysis was by Student's t-test for unpaired data.

Effect of phorbol esters and diacylglycerol on K^+ channels

The electrical properties of resting RINm5F cells, like those of normal pancreatic B cells, are dominated by two types of ATP-sensitive K^+ channels with similar properties except for the differences in unit conductance (Dunne *et al.*, 1986; Findlay *et al.*, 1985b; Petersen and Findlay, 1987). Typical membrane areas investigated in patch-clamp experiments contain 10–20 K^+ channels, but in the intact cell no more than one or two current levels can be observed due to the marked inhibition of channel opening exerted by the intracellular ATP (Dunne *et al.*, 1986; Petersen and Findlay, 1987). Ca^{2+}- and voltage-activated K^+ channels with a much higher unit conductance than the ATP-sensitive K^+ channels are also present in the RINm5F cell membrane (Findlay *et al.*, 1985a,b), but these channels are not operational in intact resting cells except transiently at the beginning of large depolarizing voltage jumps (Findlay *et al.*, 1985c) and then only when Ca^{2+} is present on the membrane outside (Velasco and Petersen, 1987). In the experiments presented here Ca^{2+} was always absent from the pipette solution and there are therefore no Ca^{2+}-activated K^+ channel openings. Glyceraldehyde depolarizes RINm5F cells by closing the ATP-sensitive K^+ channels (Dunne *et al.*, 1986). To investigate whether the action of glyceraldehyde could be mediated by diacylglycerol-evoked activation of protein kinase C we used exogenous stimuli of the kinase and monitored single K^+ channel currents.

First, the phorbol ester PMA was added to cells during patch clamp experiments conducted in the cell-attached configuration. Figure 1 shows plots of the single-channel current amplitudes for both the large and the small ATP-sensitive K^+ channels, as a function of pipette voltage before, during and after exposure of the cells to PMA (10^{-7} M). PMA caused a parallel shift to the left of the curves which was fully reversible. It also decreased the pipette voltage at which the current polarity reversed from −62 mV to −43 mV (16 experiments). Assuming that the intracellular K^+ concentration is equal to that in the pipette solution (140 mM), PMA reduced the membrane potential from −62 to −43 mV. In contrast, a PMA analogue which does not activate protein kinase C, 4-α-phorbol 12,13-didecanoate (αPDD) (10^{-7} M) had no effect on the current–voltage relations with identical reversal potentials at −68 mV in the absence and presence of the drug (14 experiments) (Figure 1). PMA at a lower concentration (10^{-8} M) evoked effects similar to those at 10^{-7} M with an average depolarization of 23 mV (17 experiments).

The depolarizing effect of PMA (10^{-8} M) was confirmed in experiments using the tight-seal whole-cell recording configuration in the current-clamp mode by adding phorbol esters prior to the penetration of the membrane. The control membrane potential at zero transmembrane current was −59 ± 3 mV (SD) and this was reduced to −31 ± 6 mV by PMA 10^{-8} M (n = 7) whereas 10^{-7} M αPDD failed to evoke membrane depolarization (membrane potential = −54 ± 3 and −53 ± 3 mV, respectively) (n = 10).

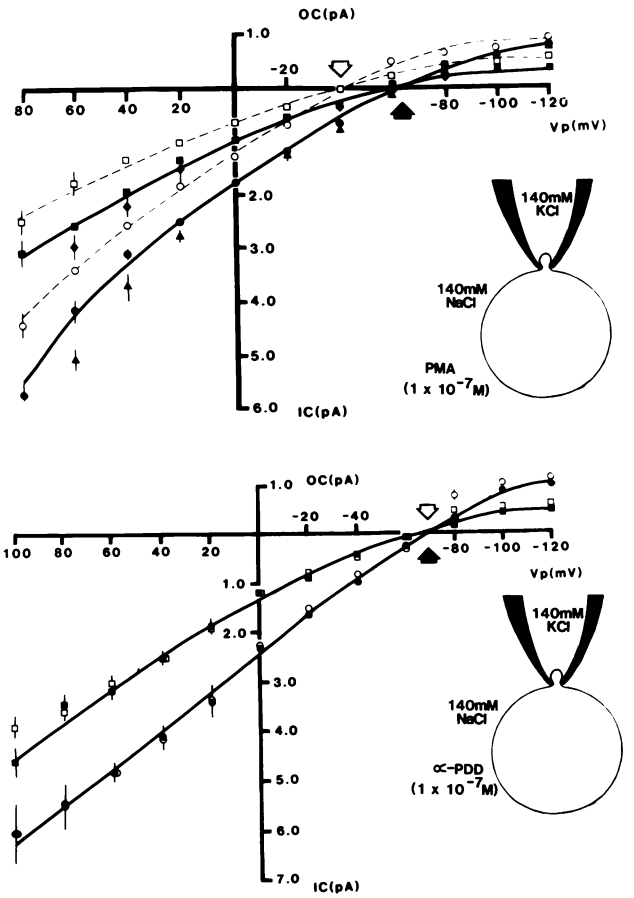

Fig. 1. Current–voltage relationships (I/V) from cell-attached membrane patches for the large and small ATP-sensitive K^+ channels (K^+ATP) in the presence and absence of 10^{-7} M PMA (**upper panel**) and 10^{-7} M αPDD (**lower panel**). Solid lines and symbols in both plots represent control values for the large and small K^+ATP channels, obtained using a Na^+-rich bathing solution (see Materials and methods). Initial control values for the large and small K^+ATP channels are represented by solid circles and solid squares, respectively, and the final control values by solid triangles and solid diamonds, respectively. In the upper panel the open circles and open squares, fitted by the dotted lines, represent the I/Vp relationships for the large and small K^+ATP channels in the presence of 10^{-7} M PMA. Mean values have been plotted from 16 experiments. In the lower panel the open symbols represent the I/Vp relationships for the large (circles) and small (squares) K^+ATP channels. Mean values have been plotted from 14 experiments. The null or reversal potential has been indicated in both plots for the control (solid arrow) and test (open arrow) situation.

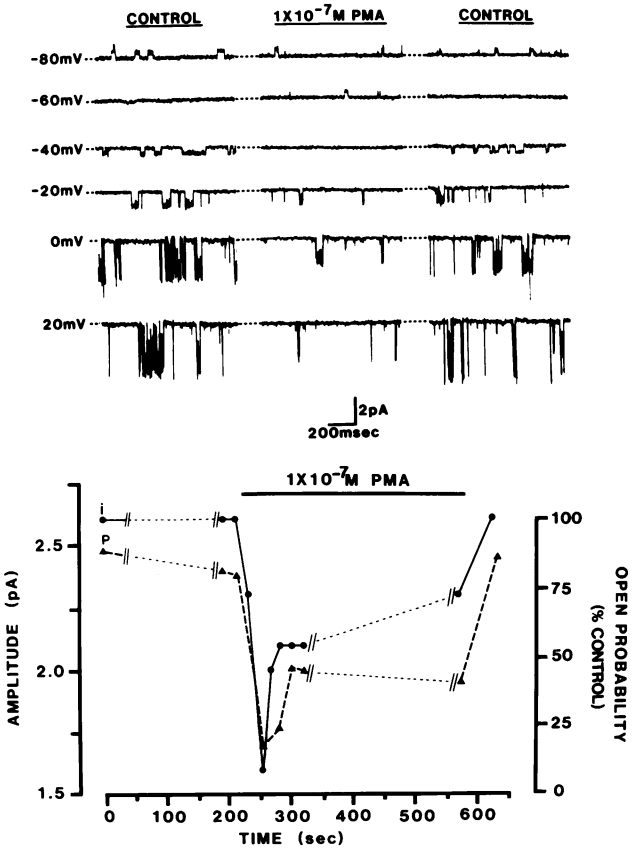

Fig. 2. Single channel current recordings obtained from a RINm5F cell-attached membrane patch showing the influence of 10^{-7} M PMA on the gating of the large K^+ATP channel. All current records in the upper panel were obtained from the same cell-attached membrane patch filtered at 500 Hz (low pass). The pipette holding potential (Vp) from which each trace was taken is illustrated on the left-hand side of the current records. Upward deflections represent current flow from the inside to the outside of the membrane patch. The initial control current records were obtained between 60 and 100 s after the formation of a giga-ohm seal and return control records between 100 and 200 s after the removal of the drug and return to control situations. Current recordings in the presence of 10^{-7} M PMA were obtained between 100 and 305 s after the acute application of the drug. The lower panel illustrates the time-course of the PMA-induced reduction in the single-channel current amplitude (*i*) (solid line) (large K^+ATP) and the reduction in channel open state probability (*P*) (dashed line) expressed as a percentage of the initial control value rather than an absolute value, since the number of ATP-sensitive K^+ channels in this membrane patch is unknown [there are always many ATP-sensitive K^+ channels in individual patch membranes, but in the intact cell channel opening is markedly inhibited by the intracellular ATP (Petersen and Findlay, 1987)]. All data points were obtained at Vp = 0 mV. The two periods during which no data are shown (between 50 and 180 s and between 320 and 550 s) correspond to time points in which the current–voltage relationship plots were generated.

Figure 2 shows an experiment in which only large ATP-sensitive K^+ channels were in evidence. In the upper part of Figure 2 it is seen that PMA evokes a marked reduction in the fractional open time of the channels. This effect was in part transient and reversible. PMA (10^{-7} M) evoked a reduction of open-state probability to 50% ± 8 (SD) (n = 9) of the preceding control level and in the five experiments that were long enough to allow return to the control situation the open-state probability was restored to 79% ± 12 of the initial control value. The reversal potential was changed from a pipette voltage of −60 mV in the control condition to −40 mV during stimulation with PMA and back again to −60 mV after return to the control solution reflecting a membrane depolarization of ~20 mV. This depolarization was not responsible for the reduction in open-state probability as repolarization of the patch membrane (by application of a positive pipette voltage of 20 mV during exposure of the cells to PMA) failed to increase the open-state probability (Figure 2 upper part). The depolarization is most likely caused by the closure of the K^+ ATP channels. The lower part of Figure 2 shows the time course of the PMA effect. Initially there is a sharp reduction in both single-channel current amplitude (*i*) (at Vp = 0 mV) and open-state probability (*P*) with the peak reduction attained ~30 s after start of stimulation, followed by partial reversal of both effects. After removal of PMA, both *i* and *P* return to the pre-stimulation control levels. In the experiment shown in Figure 2, 10^{-7} M PMA did not evoke action potentials; they were only seen in three out of the 13 experiments carried

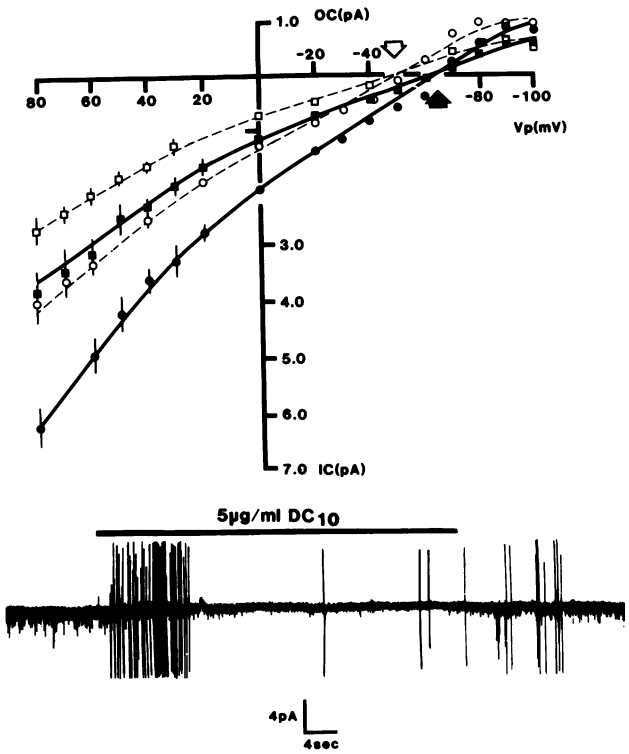

Fig. 3. Stimulation of RINm5F cells by DC_{10}. **Upper panel** shows current–voltage relationship plots obtained in cell-attached membrane patches. Solid lines and symbols represent the I/Vp relationship for the large (circles) and small (squares) K^+ATP channels in the initial control states. Dotted lines with open symbols (circles and squares) represent the I/Vp relationship for the K^+ATP channels, large and small, respectively in the presence of 5 µg/ml DC_{10} (Avanti Polar Lipids, Birmingham, AL). Null potentials for control and test solution, closed and open arrow, respectively, have been indicated. **Lower panel** shows continuous current recording trace from a cell-attached membrane patch exposed acutely to 5 µg/ml DC_{10} (bar). The pipette contained the K^+-rich solution and the bath the Na^+-rich solution (see Materials and methods). The appearance of action potentials almost immediately after the introduction of DC_{10} is clearly seen.

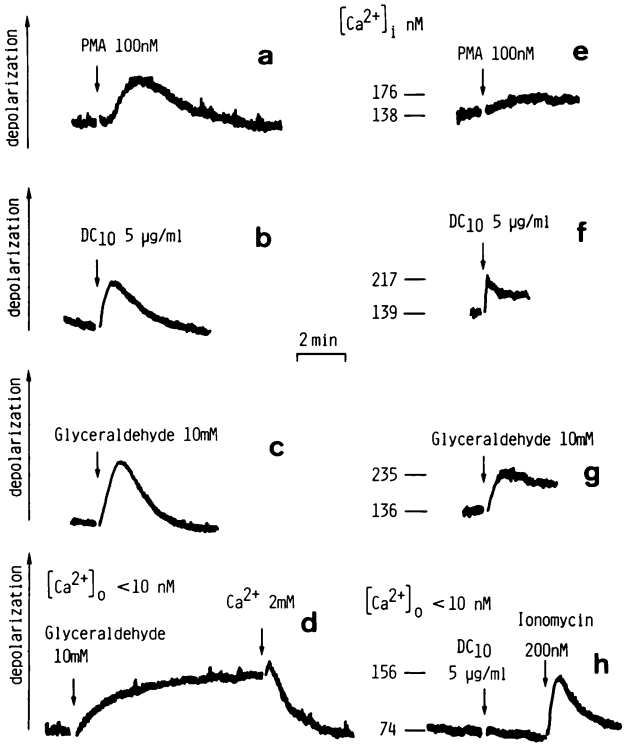

Fig. 4. Effect of PMA, DC_{10} and D-glyceraldehyde on membrane potential (**left**) and $[Ca^{2+}]_i$ (**right**) in RINm5F cells. The cells were suspended in KRBH (see Materials and methods) containing 1 mM Ca^{2+}, except for d and h, where nominally Ca^{2+}-free buffer implemented with 1 mM EGTA was used. Additions were made to yield final concentrations, except for d, where Ca^{2+} addition resulted in ~1 mM final cation concentration. Membrane potential was measured with bisoxonol and $[Ca^{2+}]_i$ on fura-2 loaded cells (see Materials and methods). The traces are representative of at least four independent experiments in each case.

out. PMA (10^{-7} M) had no direct effect on the K^+ channels as its addition to excised inside-out patches or saponin-permeabilized cells (Dunne *et al.*, 1986), in the absence of ATP and Ca^{2+}, did not cause channel closure (in eight patches 14 applications were negative, one evoked a small inhibition, one an activation).

Figure 3 shows the result of an experiment in which the synthetic diacylglycerol DC_{10} (Lapetina *et al.*, 1985) (5 µg/ml) evoked action potentials. These Ca^{2+} spikes originate outside the isolated patch area (there is no Ca^{2+} in the patch pipette solution) and occur in a short burst almost immediately after start of stimulation. Later, isolated action potentials appear. After discontinuation of DC_{10} stimulation there are a few very brief bursts of action potentials and then electrical activity ceases. DC_{10}-evoked action potentials occurred in eight out of 11 experiments. DC_{10} also caused a reduction in the open-state probability of the K^+ channels to ~47% of the control value (not shown) and evoked a leftward shift of the I/Vp curves corresponding to a depolarization of ~17 mV (Figure 3). This depolarization was also confirmed in whole-cell zero-current membrane potential measurements, where the potential was −58 ± 1 mV in the control situation and −40 ± 3 mV during exposure to DC_{10} (n = 18).

Stimulus-induced changes in membrane potential, $[Ca^{2+}]_i$ and insulin secretion

The transient depolarization response to PMA, a drug which causes long-lasting activation of protein kinase C, may appear surprising. It was, however, confirmed by measuring membrane potential in RINm5F cell suspensions with the fluorescent probe bisoxonol (Wollheim and Pozzan, 1984). Indeed, all three stimuli used, PMA, DC_{10} and glyceraldehyde evoked a similar transient membrane depolarization (Figure 4a,b and c). In the absence of extracellular Ca^{2+} all three agents caused a long-lasting depolarization. This is illustrated for glyceraldehyde in Figure 4d. Upon reintroduction of Ca^{2+} the membrane repolarized. This result suggested that a rise in $[Ca^{2+}]_i$ (see below) is mediating the repolarization of the membrane potential.

RINm5F cells possess voltage-dependent Ca^{2+} channels (Wollheim and Pozzan, 1984; Findlay and Dunne, 1985; Velasco *et al.*, 1988). Channel blockade by verapamil attenuates depolarization-mediated rises in $[Ca^{2+}]_i$ (Wollheim and Biden, 1986a; Wollheim and Pozzan, 1984; Prentki and Wollheim, 1984). PMA, DC_{10} and glyceraldehyde raised $[Ca^{2+}]_i$ measured with the fluorescent Ca^{2+} indicator fura-2 (Wollheim and Biden, 1986b; Grynkiewicz *et al.*, 1985), albeit to a varying degree (Figure 4e,f and g). The average resting $[Ca^{2+}]_i$ in these experiments was 145 ± 5 nM (mean ± SEM) (n = 39). The mean incremental increase

Table II. ATP levels in RINm5F cells stimulated with D-glyceraldehyde or PMA

Incuabtion time (min)	−2	0	1	2	3	5
			ATP (mM)			
D-Glyceraldehyde (10 mM)	3.34 ± 0.30	3.45 ± 0.21	3.55 ± 0.26	4.15 ± 0.39	3.88 ± 0.25	3.93 ± 0.19
PMA (10^{-7} M)	4.33 ± 0.15	4.59 ± 0.17	4.33 ± 0.46	4.08 ± 0.25	4.51 ± 0.32	4.57 ± 0.33

RINm5F cells were incubated and ATP measured as described in Materials and methods. The stimuli were added at 0 time. Results are given as mean ± SEM of six and three independent experiments for glyceraldehyde and PMA, respectively.

in $[Ca^{2+}]_i$ was 34 ± 8 (n = 7), 133 ± 15 (n = 13) and 99 ± 9 nM (n = 7) for PMA, DC_{10} and glyceraldehyde, respectively. The addition of verapamil (50 μM) prior to the stimuli reduced by 50−70% the rise in $[Ca^{2+}]_i$ due to PMA, DC_{10} and glyceraldehyde (n = 5−7, not shown). In the absence of extracellular Ca^{2+} none of the stimuli raised $[Ca^{2+}]_i$, which is illustrated for DC_{10} in Figure 4h. This demonstrates that the rise in $[Ca^{2+}]_i$ is secondary to stimulation of Ca^{2+} influx. However, as suggested previously for glyceraldehyde (Wollheim and Pozzan, 1984; Prentki and Wollheim, 1984), Ca^{2+} influx occurs both via verapamil-sensitive and -insensitive conductance pathways. The small rise of $[Ca^{2+}]_i$ following PMA depolarization is probably due to concomitant inhibition of voltage-dependent Ca^{2+} channels (Di Virgilio *et al.*, 1986). The larger rise in $[Ca^{2+}]_i$ observed with DC_{10} is also reflected by the induction of action potentials (see Figure 3) which are also seen following stimulation with glyceraldehyde (Dunne *et al.*, 1986).

Glucose-stimulated insulin secretion in pancreatic islets is strictly calcium dependent (Prentki and Wollheim, 1984). This is also the case for glyceraldehyde-induced insulin secretion in RINm5F cells (Wollheim and Pozzan, 1984) which do not recognize glucose as a secretagogue (Wollheim and Biden, 1986a; Prentki and Wollheim, 1984). Likewise, DC_{10} (5 μg/ml), which stimulated insulin release from 20.5 ± 1.4 to 33.9 ± 2.1 ng/10^6 cells/10 min (mean ± SEM) (n = 20) in the presence of Ca^{2+}, was ineffective in the absence of Ca^{2+}. In the same series of experiments PMA (10^{-8} M) stimulted secretion by 181%, but in contrast to DC_{10} was still partially active in Ca^{2+}-free medium (76% increase, n = 19). In pancreatic islets both PMA (Virji *et al.*, 1978; Malaisse *et al.*, 1983; Bozem *et al.*, 1987) and oleoyl-acetylglycerol (OAG) (Malaisse *et al.*, 1985) stimulate insulin secretion. Like DC_{10} in RINm5F cells, OAG-induced secretion was Ca^{2+} dependent (Malaisse *et al.*, 1985), while PMA remained partially effective in Ca^{2+}-free medium (Bozem *et al.*, 1987). In permeabilized RINm5F cells PMA is capable of eliciting insulin secretion in the complete absence of Ca^{2+}, suggesting that at least part of the effect is not due to changes in transmembrane ion fluxes (Vallar *et al.*, 1987).

There is also a difference between OAG and PMA with respect to $^{86}Rb^+$ efflux from islets. At non-stimulatory glucose concentrations, OAG reduced the efflux of Rb^+ (Malaisse *et al.*, 1985), whereas PMA did this with a retarded action in one study (Bozem *et al.*, 1987) but was ineffective in another (Malaisse *et al.*, 1983). Although a decrease in Rb^+ efflux usually reflects membrane depolarization (Henquin and Meissner, 1984), PMA failed to depolarize the B cell at non-stimulatory glucose but enhanced glucose-evoked electrical activity (Bozem *et al.*, 1987; Pace and Goldsmith, 1985). Islet B-cell membrane potential has not been monitored after exposure to OAG. There is no obvious explanation for the failure of PMA to cause B-cell depolarization by itself, while being active in RINm5F cells. It is possible that the very lipophilic compound is not evenly distributed in whole islets, which are agglomerates of 3000−5000 cells, and where the microimpalement is usually performed in the centre of the islet.

Lack of effects of PMA and glyceraldehyde on cellular ATP levels

Increases in cellular ATP levels have been suggested to mediate the closure of K^+ channels following the exposure of B cells to carbohydrates (Ashcroft *et al.*, 1984; Dunne *et al.*, 1986; Misler *et al.*, 1986; Petersen and Findlay, 1987). Therefore, we also assessed changes in ATP concentrations in RINm5F cells treated with PMA or glyceraldehyde under the same conditions as those used for the measurements of $[Ca^{2+}]_i$ and bisoxonol fluorescence, i.e. in the fluorimeter cuvette at 37°C (Table II). At 1 min, the time of maximal depolarization, glyceraldehyde did not change ATP levels, and a delayed 20% increase was not significant. PMA failed to alter ATP levels. It is thus unlikely that the effect of PMA (Figures 1 and 2) and glyceraldehyde (Dunne *et al.*, 1986) on K^+ channel conductance is mediated by changes in ATP levels. Present techniques do not allow measurement of ATP concentrations in the vicinity of the K^+ channel.

Compartmentalization of ATP has been suggested to be of importance for the regulation of cardiac ATP-sensitive K^+ channels. It was thus proposed that ATP derived from glycolysis is more effective than that derived from oxidative phosphorylation in preventing the opening of the channels (Weiss and Lamp, 1987). Such compartmentalization is not likely to occur in B cells, as ketoisocaproic acid also inhibits the K^+ channel (Ashcroft *et al.*, 1987). This secretagogue is not catabolized by glycolysis but rather oxidized in the mitochondria.

Conclusions

Phorbol esters have been shown previously to block K^+ currents in other cell types (Higashida and Brown, 1986; for review, see Kaczmarek, 1987). Our work indicates that activation of protein kinase C reduces the open-state probability of the ATP-sensitive K^+ channels. If, as would be expected, this depolarizing effect is mediated by phosphorylation of the K^+ channel, the subsequent repolarization could be due to channel reactivation upon dephosphorylation.

Alternatively, the repolarization could be due to Ca^{2+}-dependent activation of high conductance K^+ channels (Findlay *et al.*, 1985; Velasco and Petersen, 1987). We cannot distinguish between these two possibilities, since

the present studies were performed with Ca^{2+}-free solutions in the patch pipette, a condition not allowing channel activation (Velasco and Petersen, 1987).

In conclusion, we propose the following sequence of events in carbohydrate-induced insulin secretion. Glucose (Peter-Riesch *et al.*, 1988; Dunlop and Larkins, 1985) and glyceraldehyde (Table I) are rapidly metabolized to 1,2-diacylglycerol via glycolytic intermediates and phosphatidic acid. The ensuing activation of protein kinase C due to increased diacylglycerol levels leads to closure of the ATP-sensitive K^+ channels, in turn resulting in membrane depolarization, since these channels govern the resting membrane potential of insulin-secreting cells (Ashcroft *et al.*, 1984; Findlay *et al.*, 1985b; Dunne *et al.*, 1986; Petersen and Findlay, 1987; Rorsman and Trube, 1985). The depolarization, maximal after ~1 min, favours Ca^{2+} influx through voltage-dependent Ca^{2+} channels, a key event in the triggering of insulin secretion.

It would now appear that the ATP-sensitive K^+ channels have multiple regulatory mechanisms. Gating of these channels is certainly controlled by both intracellular ATP and ADP (Dunne and Petersen, 1986; Dunne *et al.*, 1988; Kakei *et al.*, 1986; Petersen and Findlay, 1987) and we are now proposing an additional regulatory mechanism mediated by protein kinase C involved in carbohydrate-evoked channel closure. These same channels can also be activated by the peptide galanin, an inhibitor of insulin secretion, via a mechanism not involving a soluble intracellular messenger (Weille *et al.*, 1988). Since RINm5F cells do not respond to glucose, an obvious next step will be to test whether our scheme of events also applies to glucose stimulation of normal pancreatic B cells. It should be noted, however, that glucose and glyceraldehyde evoke similar electrical changes in pancreatic B cells measured either with microelectrodes (for review, see Petersen and Findlay, 1987) or the patch-clamp technique (Misler *et al.*, 1986).

Materials and methods

RINm5F cells were cultured and prepared as previously described (Dunne *et al.*, 1986; Wollheim and Pozzan, 1984; Wollheim and Biden, 1986b). All single-channel current recordings were obtained in the cell-attached membrane patch configuration, using the methods described by Hamill *et al.* (1981). Patch-clamp pipettes had a final resistance between 5 and 10 Mohm when filled with a K^+-rich solution that contained (mM): 140 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 Hepes, 2.5 glucose and 0.5 EGTA, pH was set at 7.2 (KOH). RINm5F cells were bathed in a Na^+-rich solution containing (mM): 140 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 2.5 glucose and 10 Hepes, with the pH set at 7.2 (NaOH). The osmolality of all solutions was 290 ± 5 mosmol/kg. Stock solutions of PMA and αPDD were made in dimethyl sulphoxide (DMSO) and added to the Na^+ solution to give a final concentration ranging from 10^{-8} to 10^{-7} M yielding a final DMSO concentration of 0.01%. The effects of PMA and αPDD were studied using one of two experimental protocols. Either the drugs were added after establishment of the giga-seal (acute application) by the use of a continuous superfusion system from a series of outlet pipes (Dunne *et al.*, 1986) or before the pipette attachement (pre-incubation for 2–25 min). These two protocols gave the same results as far as the single-channel current amplitudes were concerned and in Figure 1 data from both protocols have been pooled. Open-state probability (*P*) estimations were from continuous stretches of current records lasting between 15 and 30 s and analysed as previously described (Dunne *et al.*, 1986). Measurements of *P* were restricted to those experiments involving acute application of PMA.

For the measurement of $[Ca^{2+}]_i$ and membrane potential with fluorescent probes, as well as cellular ATP levels and insulin secretion, suspensions of RINm5F cells were used after a 3-h spinner culture (Wollheim and Pozzan, 1984; Wollheim and Biden, 1986b). Approximately 2×10^7 cells were loaded with the Ca^{2+} indicator fura-2 by exposure for 30 min to its acetoxymethylester (1 μM) as described previously (Wollheim and Biden, 1986b). After loading, batches of $\sim 1.5 \times 10^6$ cells were resuspended in a cuvette containing 2.5 ml of a modified Krebs–Ringer–bicarbonate buffer (KRBH) with 5 mM $NaHCO_3$, 1 mM $CaCl_2$ and 2.8 mM glucose (Wollheim and Pozzan, 1984; Wollheim and Biden, 1986b). Fluorescence of fura-2-loaded cells was measured at 37°C under continuous stirring using excitation and emission wavelengths of 340 and 505 nm, respectively. $[Ca^{2+}]_i$ was calibrated after lysing the cells with 0.05% Triton X-100 and measuring fura-2 fluorescence at 1 mM and 1 nM Ca^{2+} and by using a K_d value of 225 nM (Wollheim and Biden, 1986b; Grynkiewicz *et al.*, 1985). Membrane potential fluctuations were measured by adding the fluorescent probe bisoxonol (100 nM) to the cuvette from a 1000-fold concentrated stock solution. Here the excitation wavelength was 540 nm and the emission wavelength was 580 nm (Wollheim and Pozzan, 1984; Wollheim and Biden, 1986b). Immunoreactive insulin secretion was measured during a 10-min incubation using $\sim 0.5 \times 10^6$ cells/ml exactly as described previously (Wollheim and Pozzan, 1984).

ATP levels in RINm5F cells were measured under the same conditions as $[Ca^{2+}]_i$ and bisoxonol fluorescence. To this end 1.5×10^7 cells were pre-incubated at 37°C under continuous stirring for 30 min in a cuvette containing 2 ml of the KRBH buffer (2.8 mM glucose). After addition of the stimuli at zero time, 100-μl aliquots were removed at the indicated times and transferred to ice-cold trichloroacetic acid (5%). After sonication of the cells ATP was measured in triplicate determinations by the firefly luciferase luminescence assay essentially as described by Ashcroft *et al.* (1973). The results were expressed in mM using a value of 1 μl/10^6 cells for cell water (Trautmann and Wollheim, 1987).

1,2-Diacylglycerol mass and *de novo* synthesis was measured as follows. After the spinner culture, batches of $\sim 10^7$ cells were resuspended in 1 ml of KRBH containing 2.8 mM glucose and 0.07% bovine serum albumin. Following a 10-min pre-incubation the cells were exposed to glyceraldehyde for 2 or 5 min at 37°C. The incubation was terminated by adding 10 ml of ice-cold chloroform:methanol:H_2O (2:2:1), the lipid phase was washed, dried down under nitrogen and after reconstitution in chloroform:methanol:H_2O (75:25:2) 1,2-diacylglycerols were separated from other lipids by TLC on silica G plates developed in benzene:ether:ethanol:acetic acid (25:20:1:0.1). Neutral lipids were identified from co-migrating standards after staining with rhodamine 6G. The appropriate spot was scraped off and transmethylation was performed in methanol:sulphuric acid (100:1) at 70°C for 3 h. The fatty acid esters were extracted with petroleum ether, the samples dried and reconstituted in isooctane. Then an aliquot of the sample was chromatographed on a 200 cm glass column and analysed with a Carlo Erba gas chromatograph equipped with a flame ionization detector. The gas chromatograph was calibrated using a standard mixture of methylated fatty acids. The methods are modified from published work (Banschbach *et al.*, 1981; Cockcroft and Allan, 1984) and are described in detail elsewhere (Peter-Riesch *et al.*, 1988).

For the measurements of *de novo* synthesis of 1,2-diacylglycerol, $\sim 10^7$ cells/tube were pre-incubated for 10 min in 0.5 ml of the same buffer as used for the measurement of diacylglycerol mass. Then 10 μl containing [^{14}C(U)]D-glyceraldehyde, 30 μCi per μmol (American Radiolabeled Chemicals Inc., St Louis, MO), was added to yield a final concentration of 1 mM. Incubation was stopped at the indicated times and cells processed for the separation of diacylglycerols as described above. After scraping the samples off the thin layer plates, they were diluted wth 1 ml H_2O and 10 ml Hydroluma was added for liquid scintillation spectrometry.

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References

Arkhammer,P., Nilsson,T., Rorsman,P. and Berggren,P.-O. (1987) *J. Biol. Chem.*, **262**, 5448–5454.

Ashcroft,F.M., Harrison,D.E. and Ashcroft,S.J.H. (1984) *Nature*, **312**, 446–448.

Ashcroft,F.M., Ashcroft,S.J.H. and Harrison,D.E. (1987) *J. Physiol.*, **385**, 517–529.

Ashcroft,S.J.H., Weerasinghe,L.C.C. and Randle,P.J. (1973) *Biochem. J.*, **132**, 223–231.

Banschbach,M.W., Geisen,R.L. and Hokin-Neaverson,M. (1981) *Biochim. Biophys. Acta*, **663**, 34–45.

Biden,T.J., Peter-Riesch,B., Schlegel,W. and Wollheim,C.B. (1987) *J. Biol. Chem.*, **262**, 3567–3571.

Bozem,M., Nenquin,M. and Henquin,J.C. (1987) *Endocrinology*, **121**, 1025–1033.
Cockroft,S. and Allan,D. (1984) *Biochem. J.*, **222**, 557–559.
Cook,D. and Hales,N. (1984) *Nature*, **311**, 271–273.
Di Virgilio,F., Pozzan,T., Wollheim,C.B., Vicentini,L.M. and Meldolesi,J. (1986) *J. Biol. Chem.*, **261**, 32–35.
Dunlop,M.E. and Larkins,R.C. (1985) *Biochem. Biophys. Res. Commun.*, **132**, 467–473.
Dunne,M.J. and Petersen,O.H. (1986) *FEBS Lett.*, **208**, 59–62.
Dunne,M.J., Findlay,I., Petersen,O.H. and Wollheim,C.B. (1986) *J. Membr. Biol.*, **93**, 271–279.
Dunne,M.J., Findlay,I. and Petersen,O.H. (1988) *J. Membr. Biol.*, **102**, 205–216.
Farese,R.V., DiMarco,P.E., Barnes,D.E., Sabir,M.A., Larson,L.E., Davis,J.S. and Morrison,A.D. (1986) *Endocrinology*, **118**, 1498–1503.
Findlay,I. and Dunne,M.J. (1985) *FEBS Lett.*, **189**, 281–285.
Findlay,I., Dunne,M.J. and Petersen,O.H. (1985a) *J. Membr. Biol.*, **83**, 169–175.
Findlay,I., Dunne,M.J. and Petersen,O.H. (1985b) *J. Membr. Biol.*, **88**, 165–172.
Findlay,I., Dunne,M.J., Ullrich,S., Wollheim,C.B. and Petersen,O.H. (1985c) *FEBS Lett.*, **185**, 4–8.
Grynkiewicz,G., Poenie,M. and Tsien,R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.
Hamill,O.P., Marty,A., Neher,E., Sakmann,B. and Sigworth,F.J. (1981) *Pflügers Arch.*, **391**, 85–100.
Henquin,J.C. and Meissner,H.P. (1984) *Experientia*, **40**, 1043–1052.
Higashida,H. and Brown,D.A. (1986) *Nature*, **323**, 333–335.
Kaczmarek,L.K. (1987) *Trends Neurosci.*, **10**, 30–34.
Kakei,M., Kelly,R.P., Ashcroft,F.M. and Ashcroft,S.J.H. (1986) *FEBS Lett.*, **208**, 63–66.
Lapetina,E.G., Reep,B. Ganong,B.R. and Bell,R.M. (1985) *J. Biol. Chem.*, **260**, 1358–1361.
Lernmark,Ä. (1985) *Diabetologia*, **28**, 195–203.
Malaisse,W.J. and Sener,A. (1987) *Biochim. Biophys. Acta*, **927**, 190–195.
Malaisse,W.J., Hutton,J.C., Kawazu,S., Herchuelz,A. and Sener,A. (1979) *Diabetologia*, **16**, 331–341.
Malaisse,W.J., Lebrun,P., Herchuelz,A., Sener,A. and Malaisse-Lagae,F. (1983) *Endocrinology*, **113**, 1870–1877.
Malaisse,W.J., Dunlop,M.E., Mathias,P.C.F., Malaisse-Lagae,F. and Sener,A. (1985) *Eur. J. Biochem.*, **149**, 23–27.
Misler,S., Falke,L.C., Gillis,K. and McDaniel,M.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 7119–7123.
Nishizuka,Y. (1986) *Science*, **233**, 305–312.
Pace,C.S. and Goldsmith,K.T. (1985) *Am. J. Physiol.*, **298**, C527–C534.
Peter-Riesch,B., Fathi,M., Schlegel,W. and Wollheim,C.B. (1988) *J. Clin. Invest.*, **81**, 1154–1161.
Petersen,O.H. and Findlay,I. (1987) *Physiol. Rev.*, **67**, 1054–1116.
Prentki,M. and Wollheim,C.B. (1984) *Experientia*, **40**, 1052–1060.
Rorsman,P. and Trube,G. (1985) *Pflugers Arch.*, **405**, 305–309.
Trautmann,M.E. and Wollheim,C.B. (1987) *Biochem. J.*, **242**, 625–630.
Vallar,L., Biden,T.J. and Wollheim,C.B. (1987) *J. Biol. Chem.*, **262**, 5049–5056.
Vara,E. and Tamarit-Rodriguez,J. (1986) *Metabolism*, **35**, 266–271.
Velasco,J.M. and Petersen,O.H. (1987) *Biochim. Biophys. Acta*, **896**, 305–310.
Velasco,J.M., Petersen,J.U.H. and Petersen,O.H. (1988) *FEBS Lett.*, **231**, 366–370.
Virji,M.A.G., Steffes,M.W. and Estensen,R.D. (1978) *Endocrinology*, **102**, 706–711.
Weille,J.D., Schmid-Antomarchi,H., Fosset,M. and Lazdunski,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1312–1316.
Weiss,J.N. and Lamp,S.T. (1987) *Science*, **238**, 67–69.
Wollheim,C.B. and Biden,T.J. (1986a) *Ann. N.Y. Acad. Sci.*, **488**, 317–333.
Wollheim,C.B. and Biden,T.J. (1986b) *J. Biol. Chem.*, **261**, 8314–8319.
Wollheim,C.B. and Pozzan,T. (1984) *J. Biol. Chem.*, **259**, 2262–2267.
Wollheim,C.B., Ullrich,S. and Pozzan,T. (1984) *FEBS Lett*, **177**, 17–22.

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