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Okadaic acid-induced decrease in the magnitude and efficacy of the Ca^{2+} signal in pancreatic β cells and inhibition of insulin secretion

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1 Phosphorylation by kinases and dephosphorylation by phosphatases markedly affect the biological activity of proteins involved in stimulus-response coupling. In this study, we have characterized the effects of okadaic acid, an inhibitor of protein phosphatases 1 and 2A, on insulin secretion. Mouse pancreatic islets were preincubated for 60 min in the presence of okadaic acid before their function was studied.

2 Okadaic acid dose-dependently ($IC_{50} \sim 200$ nM) inhibited insulin secretion induced by 15 mM glucose. At 0.5 μM, okadaic acid also inhibited insulin secretion induced by tolbutamide, ketoisocaproate and high K^+, and its effects were not reversed by activation of protein kinases A or C.

3 The inhibition of insulin secretion did not result from an alteration of glucose metabolism (estimated by the fluorescence of endogenous pyridine nucleotides) or a lowering of the ATP/ADP ratio in the islets.

4 Okadaic acid treatment slightly inhibited voltage-dependent Ca^{2+} currents in β cells (perforated patch technique), which diminished the rise in cytoplasmic Ca^{2+} (fura-2 method) that glucose and high K^+ produce in islets. However, this decrease (25%), was insufficient to explain the corresponding inhibition of insulin secretion (90%). Moreover, mobilization of intracellular Ca^{2+} by acetylcholine was barely affected by okadaic acid, whereas the concomitant insulin response was decreased by 85%.

5 Calyculin A, another inhibitor of protein phosphatases 1 and 2A largely mimicked the effects of okadaic acid, whereas 1-norokadaone, an inactive analogue of okadaic acid on phosphatases, did not alter β cell function.

6 In conclusion, okadaic acid inhibits insulin secretion by decreasing the magnitude of the Ca^{2+} signal in β cells and its efficacy on exocytosis. The results suggest that, contrary to current concepts, both phosphorylation and dephosphorylation of certain β cell proteins may be involved in the regulation of insulin secretion.

Keywords: Pancreatic islets; insulin secretion; okadaic acid; calyculin A; protein phosphatase inhibitors; cytoplasmic Ca^{2+}; Ca^{2+} channels

Introduction

Reversible protein phosphorylation is a major mechanism in stimulus-response coupling (Cohen, 1989, Shenolikar, 1994). In many cell types, phosphorylation and dephosphorylation of ionic channels or regulatory intracellular proteins by kinases and phosphatases, respectively, underlie the induction of specific responses by extracellular messengers.

Insulin secretion is primarily stimulated by increases in the plasma concentration of nutrients, in particular glucose, which must be metabolized by pancreatic β cells to promote release of the hormone. This metabolism activates two regulatory pathways. The major one involves closure of adenosine triphosphate (ATP)-sensitive K^+ channels (K^+-ATP channels), membrane depolarization, opening of voltage-dependent Ca^{2+} channels, influx of Ca^{2+} and rise in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) which is a triggering signal for exocytosis (Ashcroft & Rorsman, 1989; Henquin, 1994). The second pathway does not involve further changes in $[Ca^{2+}]_i$ but an increase in the efficacy of Ca^{2+} on exocytosis, through an as yet unidentified mechanism (Gembal *et al*., 1992; 1993; Sato *et al*., 1992). Insulin secretion is also under strong hormonal and neural control. Acetylcholine, catecholamines, gastro-intestinal hormones are physiological, important modulators of β cells in which they exert their effects by activating membrane receptors coupled to classical transduction pathways (Henquin, 1994; Liang & Matschinsky, 1994).

The rise in $[Ca^{2+}]_i$ with subsequent activation of Ca^{2+} calmodulin-dependent kinases and the receptor-mediated activation of protein kinases A and C lead to phosphorylation of a number of still largely unidentified proteins in islet cells (for reviews see Ashcroft, 1994; Howell *et al*., 1994). It has generally been held that increased protein-phosphorylation favours exocytosis. As the phosphorylation state is determined by the balance between the phosphorylation and dephosphorylation rates, protein phosphatases might also serve a regulatory role, and their inhibition could be expected to increase insulin secretion. This question has previously been addressed by using okadaic acid (OKA), a membrane permeant inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) (Bialojan & Takai, 1988; Hardie *et al*., 1991), but the results are controversial. In β cell lines, OKA was found to increase insulin release slightly and transiently (Haby *et al*., 1994; Mayer *et al*., 1994) or to inhibit it (Zhang & Kim, 1995; Ammon *et al*., 1996). In rat islets, glucose-induced insulin release was strongly impaired by OKA (Tamagawa *et al*., 1992; Murphy & Jones, 1996). In the present study, we have explored the influence of OKA on various aspects of β cell function and compared its effects to those of 1-norokadaone (NOK) an inactive analogue (Nishiwaki *et al*., 1990), and calyculin A (CLA), a structurally unrelated inhibitor of PP1 and PP2A (Ishihara *et al*., 1989). The results showed that OKA inhibits

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insulin secretion by decreasing the magnitude of the Ca^{2+} signal and its efficacy on exocytosis.

Methods

Preparation and solutions

All procedures have been approved by the University animal care committee. Islets were isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), followed by hand-picking. In some experiments the islets were preincubated and then incubated immediately after isolation. In other experiments the islets were prepared aseptically and cultured for 1–2 days in RPMI 1640 medium (Gibco BRL, Paisley, Scotland) containing 10 mM glucose, 10% heat-inactivated foetal calf serum, 100 iu ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For patch-clamp experiments, the islets were dispersed into single cells with Ca^{2+} free buffer containing 100 μM EGTA and 100 μg ml^{-1} trypsin. The cells were then cultured for 1–2 days.

The medium used for islet isolation and testing was a bicarbonate-buffered solution that contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 24 mM $NaHCO_3$. It was gassed with $O_2:CO_2$ (94:6) to maintain pH 7.4 and was supplemented with bovine serum albumin (1 mg ml^{-1}). Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$. When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly to maintain isoosmolarity. With the exception of the patch-clamp recordings, which were performed at room temperature, all experiments were carried out at 37°C.

Measurement of insulin secretion

In the first type of experiment (static incubations), freshly isolated islets were preincubated for 60 min in a control medium containing 3 mM glucose before being distributed into batches of three. When the ATP and ADP contents were to be measured simultaneously, the islets were distributed into batches of five. Each batch of islets was then incubated for 60 min in 1 ml of medium containing various concentrations of glucose and test substances. A portion of the medium was withdrawn at the end of the incubation and appropriately diluted for insulin assay. Insulin was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark). In one series, the islets were cultured for 45 h with or without 200 ng ml^{-1} pertussis toxin before their function was tested during incubations.

In another type of experiment (dynamic perifusions), cultured islets were preincubated for 60 min in a control medium containing 10 mM glucose before being placed, in batches of 20–25, in parallel perifusion chambers and perifused at a flow rate of 1.25 ml min^{-1} (Henquin, 1978). Effluent fractions were collected at 1–2 min intervals and insulin was measured as above.

Measurement of islet ATP and ADP

These determinations were made at the end of incubations that also served to study insulin secretion (see above). After the aliquot (0.625 ml) for insulin measurement was taken, the islets were incubated for another 5 min. The tubes were kept at 37°C during the whole procedure. The incubation was then stopped by addition of 0.125 ml of trichloroacetic acid to a final concentration of 5%. The tubes were then treated, and ATP and ADP contents of the islets were measured, as published recently (Detimary *et al*., 1995).

Measurement of cytoplasmic Ca^{2+}

Cultured islets were loaded with 2 μM fura PE3/AM (Mobitec, Göttingen, Germany) during 120 min of incubation at 37°C in a bicarbonate-buffered solution containing 3 or 10 mM glucose. The islets were then transferred into a temperature-controlled perifusion chamber (Intracell, Royston, Herts, UK) with a bottom made of a coverslip and mounted on the stage of an inverted microscope. The islets were held in place by gentle suction with a glass micropipette. The preparation was perifused at a flow rate of 1.3 ml min^{-1}. The dead space of the system (2 min) has been corrected for in figures and calculations. Perifusion solutions were kept at 38°C in a water bath and the temperature controller ensured a temperature of 37°C ($\pm$ 0.3°C) in the chamber as monitored by a thermistor placed near the tissue.

The measurements of $[Ca^{2+}]_i$ were performed with the system MagiCal (Applied Imaging, Sunderland, U.K.) as described in detail (Gilon & Henquin, 1992). The tissue was excited at 340 nm and 380 nm. The fluorescence emitted at 510 nm was captured by a CCD video camera (Photonic Science Ltd, Tunbridge Wells, U.K.). From the ratio of the fluorescence at 340 and 380 nm, the concentration of $[Ca^{2+}]_i$ was calculated by comparison with a calibration curve (Gilon & Henquin, 1992).

Measurement of reduced pyridine nucleotide fluorescence

Cultured islets were first preincubated for 60 min at 37°C in control medium containing 3 mM glucose. They were then transferred to the same experimental set-up as for $[Ca^{2+}]_i$ measurements. The reduced forms of NAD and NADP, referred to as NAD(P)H, were excited at 360 nm, and the fluorescence emitted was filtered at 470 nm (Gilon & Henquin, 1992). The changes in fluorescence were expressed as a percentage of basal values by dividing the integrated gray levels at a given time by those obtained during the last min preceding stimulation with 15 mM glucose.

Measurement of Ca^{2+} currents

After culture, dispersed β cells were preincubated for 60 min at 37°C in control medium containing 10 mM glucose without or with 0.5 μM OKA or NOK. Voltage-clamp experiments were performed in the perforated-patch configuration (Horn & Marty, 1988) with an EPC-9 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). Membrane currents were recorded with the Pulse 8.05 software (HEKA Electronics). Before storage at a sampling rate of 10 kHz, they were filtered at 3 kHz with a Bessel filter. Voltage-dependent Ca^{2+} currents were corrected for leak currents with a P/n protocol (P/n pulses = 16). Depolarizations of 50 ms duration were applied from a holding potential of -80 mV.

Recordings were carried out at 22°C in an extracellular medium containing (mM) NaCl 125, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 5, glucose 15, tetraethylammonium chloride 10 and tetrodotoxin 0.1 μM. pH and osmolarity were adjusted to 7.4 and 310 mOsmol l^{-1}, respectively. The pipette solution for recording Ca^{2+} currents contained (in mM) CS_2SO_4 76, NaCl 10, KCl 10, $MgCl_2$ 1, HEPES 5 and amphotericin B 0.25 mg ml^{-1}. Osmolarity was adjusted to 290 mOsmol l^{-1} with mannitol (1 g 100 ml^{-1}) and pH was adjusted to 7.25.

Materials

Diazoxide was from Schering-Plough Avondale (Rathdrum, Ireland); okadaic acid (OKA) and calyculin A (CLA) were from Biomol (Hamburg, Germany); 1-norokadaone (NOK) was from Calbiochem (San Diego, CA); ATP, ADP, and all reagents for their measurements were from Boehringer-Mannheim (Mannheim, Germany); dibutyryl (db)-cyclicAMP and α-ketoisocaproate (KIC) were from Aldrich Chemie (Steinheim, Germany); phorbol 12-myristrate 13-acetate (PMA), tolbutamide, tetraethylammonium chloride, acetylcholine chloride (ACh), amphotericin B and pertussis toxin were from Sigma Chemical Co. (St. Louis, MO); tetrodotoxin was from RBI (Natick, MA).

Presentation of results

Results are presented as means ± s.e. for the indicated number of batches of islets. The statistical significance of differences between means was assessed by Student's *t* test for unpaired data when only two groups were compared, or by analysis of variance followed by Dunnett's test for multiple comparisons. Differences were considered significant at $P<0.05$.

Results

Time-dependence of the effects of okadaic acid on insulin secretion

The islets were incubated in a control medium containing 15 mM glucose, i.e. under conditions where the primary mechanism of glucose regulation (at the K^+-ATP channel level) plays the predominant role. They were also incubated in a medium containing 250 μM diazoxide to keep K^+-ATP channels open (Sturgess *et al*., 1988) and 30 mM K^+ to depolarize the membrane, i.e. under conditions where the second mechanism of glucose regulation can be studied (Gembal *et al*., 1992). Addition of 0.5 μM OKA to the incubation medium did not significantly affect insulin secretion induced by 15 mM glucose (88 ± 11% of controls, $n=15$) and only slightly inhibited the secretion induced by the combination of 20 mM glucose and high K^+ (75 ± 5% of controls, $n=26$; $P<0.05$). We then observed that the inhibition was larger when the islets were preincubated for 60 min with OKA (Figure 1), and that this inhibition was unaffected by the persistence or omission of OKA during the subsequent incubation. This may be due to a relatively slow permeation of OKA and reflect the time needed to reach an intracellular concentration that completely inhibits phosphatases. Similar observations have been made by others (Ashizawa *et al*., 1989; Yanagihara *et al*., 1991; Zhang & Kim, 1995). In all subsequent experiments, therefore, OKA was added to the preincubation medium only.

Concentration-dependence of the effects of okadaic acid on insulin secretion

Preincubation of the islets for 60 min in the presence of various concentrations of OKA resulted in a concentration-dependent inhibition of insulin secretion induced by either 15 mM glucose or the combination of 20 mM glucose and 30 mM K^+ in the presence of diazoxide (Figure 1). The concentration-response curve was similar for both stimuli, with a first significant effect at 50 nM OKA ($P<0.05$), an estimated EC_{50} around 200 nM,

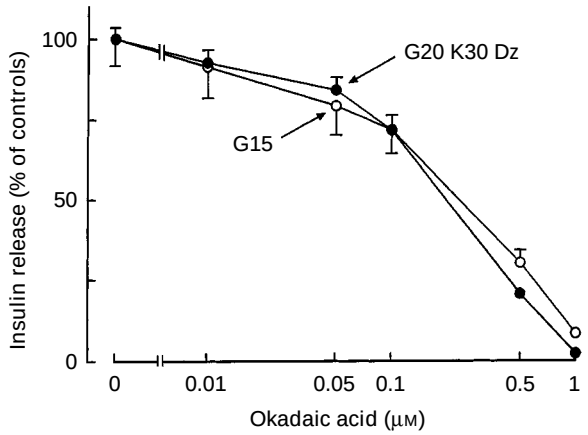

Figure 1 Concentration-dependence of the effects of okadaic acid on insulin release by incubated mouse islets. After isolation the islets were preincubated for 60 min in a medium containing 3 mM glucose and the indicated concentration of okadaic acid. They were then washed and distributed in batches of three in 1 ml of medium containing 15 mM glucose (G15) or 20 mM glucose, 30 mM K^+ and 250 μM diazoxide (G20 K30 Dz). After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means for 20 batches of islets from four separate experiments; vertical lines show s.e.mean. Test values obtained after okadaic acid treatment were expressed as a percentage of control values without okadaic acid in each experiment. Absolute values for these controls were: 3.3 ± 0.4 ng/islet 60 min^{-1} (G15 mM) and 12.9 ± 1.1 ng/islet 60 min^{-1} (G20 K30 Dz).

and abrogation of secretion by 1 μM OKA (Figure 1). On the basis of these results 0.5 μM OKA was selected for the subsequent experiments.

Comparison of the effects of okadaic acid to those of 1-norokadaone and calyculin A

As shown in Figure 2 (a,c), OKA did not affect basal insulin secretion (3 mM glucose) but inhibited the response to 15 mM glucose or to high K^+ in absence or presence of glucose. In contrast, NOK, an inactive analogue of OKA, was consistently ineffective. CLA, another type of protein phosphatase inhibitor was tested at the single concentration of 10 nM, and was found to inhibit glucose- and high K^+-induced insulin secretion by about 50% (Figure 2a,c).

Influence of okadaic acid on the energy state of the islets

Glucose metabolism and the subsequent changes in β cell energy state are critical events for the stimulation of insulin release by both control pathways (Ashcroft, 1980; Gembal *et al*., 1993; Henquin, 1994). We, therefore, tested whether they were altered by the protein phosphatase inhibitors. Raising the concentration of glucose from 3 to 15 mM accelerated β cell metabolism, as shown by the marked increase in NAD(P)H fluorescence in the islets (Figure 3). This increase was not impaired when the islets had been treated by OKA or CLA (Figure 3 – inset).

ATP and ADP concentrations were measured in batches of islets from which insulin secretion was also studied (Figure 2, b,d). In a control medium, raising the glucose concentration from 3 to 15 mM increased the ATP/ADP ratio from 2.5 ± 0.1 to 4.0 ± 0.2 ($P<0.01$). In the presence of 30 mM K^+ and diazoxide, the ATP/ADP ratio increased from 2.0 ± 0.1 in the absence of glucose to 3.7 ± 0.1 in the presence of 20 mM glucose ($P<0.01$). These changes are smaller than those we obtained recently because the present measurements were done

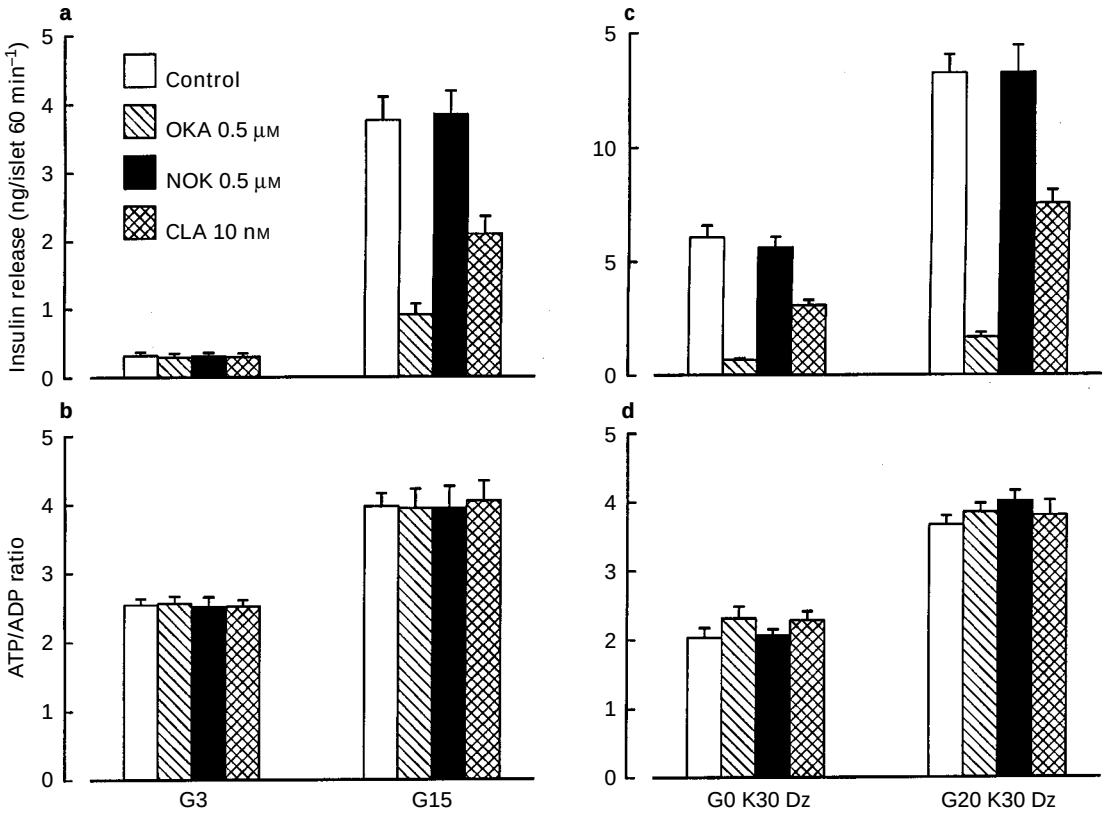

Figure 2 Effects of okadaic acid (OKA), 1-norokadaone (NOK) and calyculin A (CLA) on insulin release (a and c) and the ATP/ADP ratio (b and d) in incubated mouse islets. After isolation the islets were preincubated for 60 min in a medium containing 3 mM glucose alone or with 0.5 μM OKA, 0.5 μM NOK or 10 nM CLA. They were then incubated in the presence of 3(G3) or 15(G15) mM glucose alone, or in a medium with 30 mM K^+, 250 μM diazoxide and 0 or 20 mM glucose. (G0K30Dz and G20K30Dz, respectively). Islet ATP and ADP contents were not measured in all experiments. For insulin release values are means $\pm$ s.e.mean for 20 or 30 batches of islets (4–6 separate experiments). For the ATP/ADP ratio, values are means$\pm$s.e.mean for 15 batches of islets (3 separate experiments).

in freshly isolated, well granulated islets (Detimary *et al.*, 1995). The important observation is that neither OKA nor CLA affected the ATP/ADP ratio although they inhibited insulin secretion from the same islets (Figure 2).

Effects of okadaic acid and calyculin A on insulin secretion induced by various agents

The next series of experiments was performed to evaluate whether the inhibition of insulin secretion by OKA and CLA could be prevented by agents acting at different steps of stimulus-secretion coupling. Pharmacological closure of K^+-ATP channels by tolbutamide (Sturgess *et al.*, 1988) potentiated glucose-induced insulin release but did not prevent the inhibition by OKA or CLA (Table 1). Both drugs also inhibited insulin secretion induced by ketoisocaproate which is exclusively metabolized in mitochondria. Glucose- and high K^+-induced insulin secretion was potentiated by activation of protein kinase A with dibutyryl cyclicAMP or protein kinase C with PMA (Table 1). However, this activation of the kinases failed to reverse the inhibition by the phosphatase inhibitors. NOK was also tested in some of these experiments and consistently found to be without effect (data not shown).

Taken together the above results indicate that OKA and CLA inhibit the secretory response to agents acting differently. This suggests that the inhibitors of protein phosphatases might affect a common key step of stimulus-secretion coupling. The possible intervention of a G_i-mediated mechanism (Sharp, 1996) was tested by culturing the islets for 45 h with 200 ng ml^{-1} pertussis toxin. This reduced the inhibition of glucose-induced insulin release by 0.1 μM adrenaline from 87$\pm$1% to 23$\pm$4%, but did not influence the inhibitory effect of 0.5 μM OKA (83$\pm$2 and 80$\pm$2%; n=11). The possible influence of OKA on $[Ca^{2+}]_i$ was next evaluated.

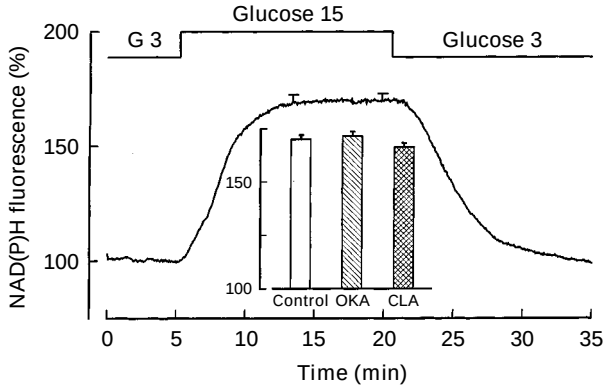

Figure 3 Effects of okadaic acid (OKA) and calyculin A (CLA) on the NAD(P)H fluorescence of glucose-stimulated mouse islets. After isolation the islets were cultured for 1–2 days before being preincubated for 60 min in a medium containing 3 mM glucose and either 0.5 μM OKA or 10 nM CLA. They were then perifused with a medium containing 3 or 15 mM glucose as indicated. CLA was also present in the perifusion medium. The results are expressed as a percentage of the fluorescence recorded in each islet in the presence of 3 mM glucose. The trace shows the response of control islets. The inset shows the mean responses between 15 and 20 min in the 3 groups of islets. Values are means$\pm$s.e.mean for 17–19 islets from 4 different cultures.

Table 1 Effects of okadaic acid and calyculin A on insulin release induced by various agents

Line	Test agents	*Insulin release* (ng/islet 60 min^{-1}) Controls	Okadaic acid	Calyculin A
	K 4.8 mM			
1	G15 mM	3.3±0.5	0.7±0.2 (21%)	1.5±0.2 (45%)
2	G15+DbcAMP 0.5 mM	9.5±1.0	2.3±0.4 (24%)	6.4±0.7 (67%)
3	G15+PMA 25 nM	17.9±1.2	3.7±0.5 (21%)	10.8±1.0 (60%)
4	G15+Tolbutamide 100 μM	10.4±1.1	1.3±0.2 (13%)	5.3±0.8 (51%)
5	G3+Ketoisocaproate 10 mM	10.5±1.0	1.5±0.2 (14%)	7.1±0.9 (68%)
	K 30 mM+diazoxide			
6	G20 mM	15.0±1.5	1.8±0.2 (12%)	9.9±1.3 (66%)
7	G20+DbcAMP 0.5 mM	22.2±2.0	3.6±0.4 (16%)	16.4±2.2 (74%)
8	G20+PMA 25 nM	26.7±1.8	5.2±0.7 (19%)	19.1±1.7 (72%)

Freshly isolated islets were preincubated for 60 min in a control medium containing 3 mM glucose (G) alone or with 0.5 μM okadaic acid or 10 nM calyculin A. They were then distributed in batches of three and incubated for 60 min in the presence of the indicated test agents. Values are means±s.e. for 15 batches of islets from at least 3 separate experiments. All values in the presence of okadaic acid or calyculin A are significantly smaller than control values.

Effects of okadaic acid on β cell cytoplasmic Ca^{2+} and Ca^{2+} currents

Stimulation of perifused islets by a rise in the glucose concentration from 3 to 15 mM evoked a biphasic release of insulin (Figure 4a). Both phases were strongly inhibited (~85%) after preincubation of the islets with 0.5 μM OKA. The changes in β cell $[Ca^{2+}]_i$ were measured in individual islets, but the responses were averaged to facilitate comparison with secretion changes. The rise in $[Ca^{2+}]_i$ induced by 15 mM glucose was slightly slower and ~25% smaller ($P<0.01$) in OKA-treated than control islets (Figure 4b). During steady state stimulation with glucose, oscillations of $[Ca^{2+}]_i$ were observed in 83% of control islets and only 59% of OKA-treated islets, and these oscillations were smaller than control ones (Figure 4c). In contrast, NOK affected neither the proportion of islets showing $[Ca^{2+}]_i$ oscillations nor the average $[Ca^{2+}]_i$ rise (Figure 4c).

Glucose-induced changes in $[Ca^{2+}]_i$ result from membrane depolarization secondary to closure of K^+-ATP channels. This indirect mechanism can be short-circuited by raising extracellular K^+ in the presence of diazoxide, which holds K^+-ATP channels open (Sturgess *et al*., 1988; Gembal *et al*., 1992). In the absence of glucose, this combination induced a marked rise in $[Ca^{2+}]_i$ that was partially inhibited (~25%) by OKA and CLA, and unaffected by NOK (Figure 5). Similar results were obtained when the experiments were carried out in the presence of 20 mM instead of 0 glucose or at 22°C instead of 37°C (data not shown).

As these results suggested that OKA and CLA inhibit depolarization-induced Ca^{2+} influx in β cells, voltage-dependent Ca^{2+} currents were directly measured (at 22°C) by the perforated patch technique, in the presence of 2.5 mM extracellular Ca^{2+}. Depolarization from a holding potential of −80 mV elicited inward Ca^{2+} currents which became detectable at −40 mV, were maximal at 0 mV and then declined at more positive voltages (Figure 6). Pretreatment of the cells with 0.5 μM OKA inhibited Ca^{2+} currents by ~30%, whereas NOK was without effect. For unknown reasons it proved impossible to form adequate seals with cells pretreated with CLA.

Inhibitory effects of okadaic acid beyond the $[Ca^{2+}]_i$ rise

The above results show that OKA inhibits Ca^{2+} entry in β cells to a much lesser extent than insulin secretion, which raises the possibility that the action of Ca^{2+} is also impaired. This was tested in two ways.

Islets perifused with a medium containing 15 mM glucose and 250 μM diazoxide were stimulated by 30 mM K^+ in the presence of 0.8 mM $CaCl_2$ (Figure 7). This resulted in a rapid increase followed by a stable elevation of $[Ca^{2+}]_i$. A subsequent rise in extracellular Ca^{2+} from 0.8 to 2.5 mM in the presence of high K^+ caused a further increase in $[Ca^{2+}]_i$. These changes were parallel in control and OKA-treated islets, but $[Ca^{2+}]_i$ consistently remained lower in the latter. Insulin secretion occurring under these conditions is shown in Figure 7a. The secretion rate increased stepwise in control islets, but was barely accelerated by high K^+ when the islets had been treated with OKA. It is striking that insulin release was much lower in OKA-treated islets stimulated by 30 mM K^+ and 2.5 mM Ca^{2+} than in control islets stimulated by 30 mM K^+ and 0.8 mM Ca^{2+} although $[Ca^{2+}]_i$ was slightly higher (218±10 nM and 197±8 nM, respectively).

In the absence of extracellular Ca^{2+}, mobilization of intracellular Ca^{2+} by 100 μM acetylcholine evoked a large peak of $[Ca^{2+}]_i$ followed by a small plateau, and triggered a peak of insulin secretion in control islets (Figure 8). Similar changes in $[Ca^{2+}]_i$ were observed in islets pretreated with OKA, but all values were slightly lower than in controls. However, the rise between basal and peak $[Ca^{2+}]_i$ was only 15% smaller in test islets (150 vs 177 nM). On the other hand, the maximum secretion rate was only 2.8 fold the basal rate in OKA-treated islets, as compared with almost 10 fold in control islets (Figure 8).

Discussion

Okadaic acid was found to inhibit insulin secretion from mouse islets in a concentration-dependent manner (IC_{50} ~200 nM). An inhibition of glucose-induced insulin secretion by OKA has also been observed in rat islets (Tamagawa *et al*., 1992; Murphy & Jones, 1996). The apparently lower sensitivity (~5 fold) of rat islets to OKA inhibition can probably be explained by the fact that the drug was present during incubation only, and not during preincubation as in our study. In the glucose-sensitive INS cell line, an inhibition of glucose-induced insulin release was also observed after preincubation with 1 μM OKA (Zhang & Kim, 1995). The results obtained with the glucose-unresponsive RINm5F cell line are less coherent. OKA was

found to cause a small and transient increase in basal $[Ca^{2+}]_i$ and insulin secretion (Haby *et al*., 1994; Mayer *et al*., 1994) or to inhibit K^+ induced $[Ca^{2+}]_i$ rise and insulin secretion (Ammon *et al*., 1996). These discrepancies may reflect differences between subclones of tumour cells.

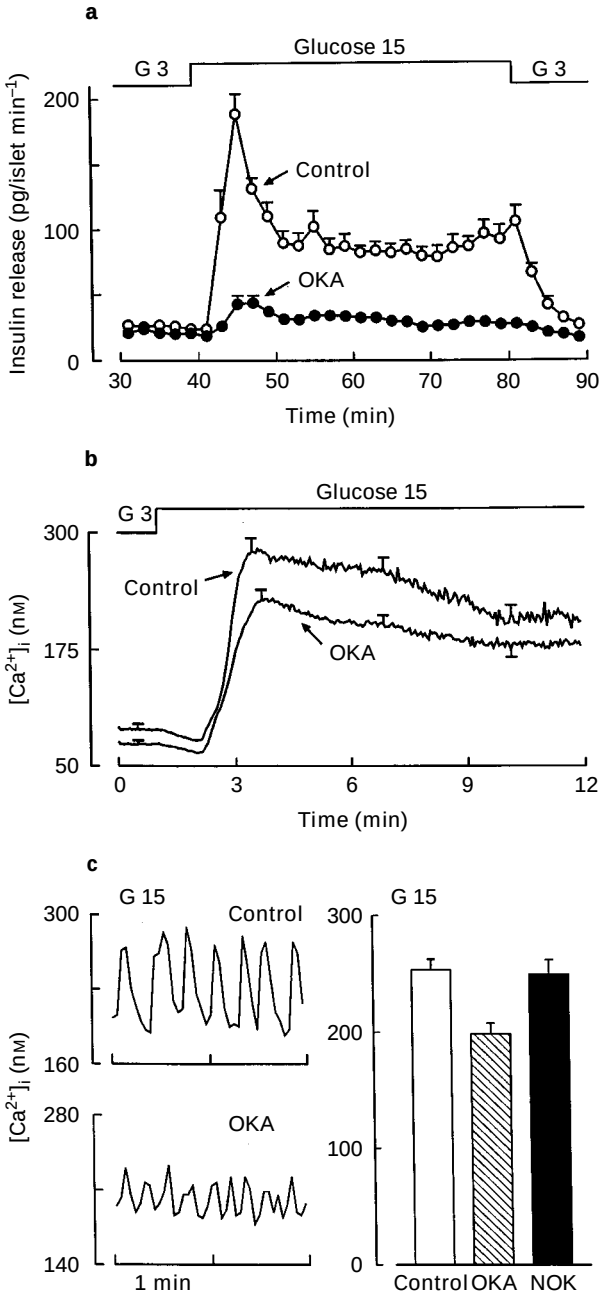

Figure 4 Effects of okadaic acid (OKA) on (a) insulin release and (b and c) $[Ca^{2+}]_i$ in glucose stimulated mouse islets. After isolation, the islets were cultured for 1 day before being preincubated for 60 min in a medium containing 10 mM glucose in the absence of presence of 0.5 μM OKA. When $[Ca^{2+}]_i$ was to be measured, the islets were also loaded with fura PE3 for 120 min, 0.5 μM OKA or 0.5 μM NOK being added during the last 60 min of this preincubation period. The measurements of insulin secretion (batches of 20 islets) and $[Ca^{2+}]_i$ (single islets) were performed in different perifusion systems. The glucose concentration of the medium was raised from 3 to 15 mM as indicated. (a) Shows mean values and s.e.mean (vertical lines) for 6 separate experiments. (b) The time course of the changes in $[Ca^{2+}]_i$ measured in 30 control and OKA-treated islets. (c) On the left are shown representative oscillations in $[Ca^{2+}]_i$ that were recorded in individual control or test islets. The average $[Ca^{2+}]_i$ between 5–7 min of stimulation with 15 mM glucose is shown on the right (means $\pm$ s.e.mean for 30 islets from 4 different cultures).

OKA is widely used as a selective inhibitor of PP1 and PP2A (Bialojan & Takai, 1988; Hardie *et al*., 1991). In cell-free systems, it is more potent on PP2A than PP1 activity (IC_{50} 0.1 and 10 nM, respectively). However, in intact cells concentrations approaching 1 μM must be used because both phosphatases are present at high concentrations (Hardie *et al*., 1991), and selective inhibition of PP1 and PP2A is not possible (Hardie *et al*., 1991). PP1 and PP2A are both present in mouse and rat islets (Ämmälä *et al*., 1994; Murphy & Jones, 1996) together with PP2B (Gagliardino *et al*., 1991; Murphy & Jones, 1996), which is much less sensitive to OKA (Bialojan & Takai, 1988). PP3, which is also inhibited by OKA, has not been detected in insulin-secreting cells (Sjöholm *et al*., 1993). In islet and RINm5F cell extracts, PP1/2A activity was inhibited by OKA over the concentration range 0.1 nM – 1 μM (Sjöholm *et al*., 1993; Ammon *et al*., 1996; Murphy & Jones, 1996). By contrast, higher concentrations of OKA had to be used to demonstrate increases in the phosphorylation of specific proteins in intact islet cells: 10 μM OKA during incubation only (Persaud *et al*., 1996) or 1 μM OKA during preincubation (Zhang & Kim, 1995). Our results obtained after preincubation with 0.5 μM OKA can thus reasonably be explained by a slow permeation of the drug and slow inhibition of protein phosphatase activity in intact cells. The lack of effect of 1-norokadaone, a classical inactive control of OKA (Nishiwaki *et al*., 1990) and the qualitatively similar effects produced by calyculin A, which is structurally unrelated to OKA but also inhibits PP1 and PP2A (Ishihara *et al*., 1989), also support this proposal. Admittedly, direct measurements of protein phos-

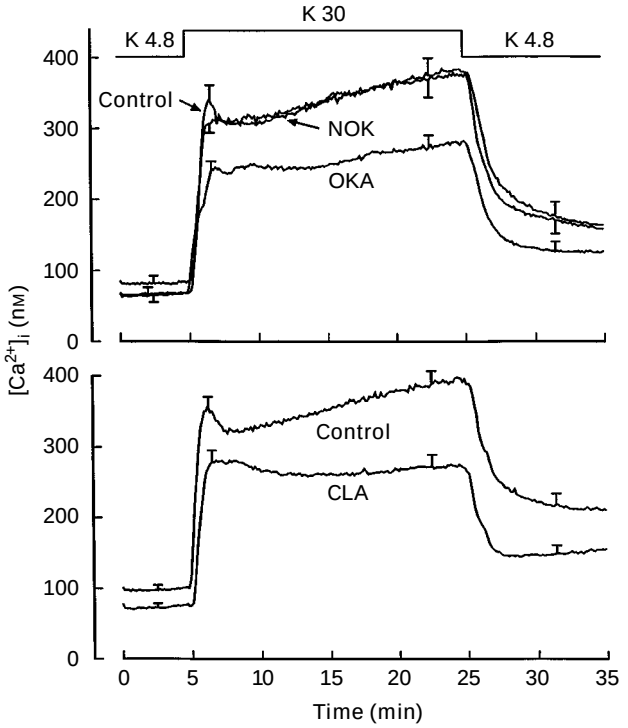

Figure 5 Effects of okadaic acid (OKA), 1-norokadaone (NOK) and calyculin A (CLA) on K^+-induced changes in $[Ca^{2+}]_i$ in perifused mouse islets. After isolation the islets were cultured for 1–2 days before being loaded with fura PE3 for 120 min. The medium contained 3 mM glucose and, for test islets, was supplemented with 0.5 μM OKA, 0.5 μM NOK or 10 nM CLA during the last 60 min. The islets were then perifused with a medium containing no glucose and 250 μM diazoxide. Between 5 and 25 min the K^+ concentration was raised from 4.8 to 30 mM. CLA was also present during the perifusion. Values are means and s.e.mean (vertical lines) for 10 islets from two separate cultures.

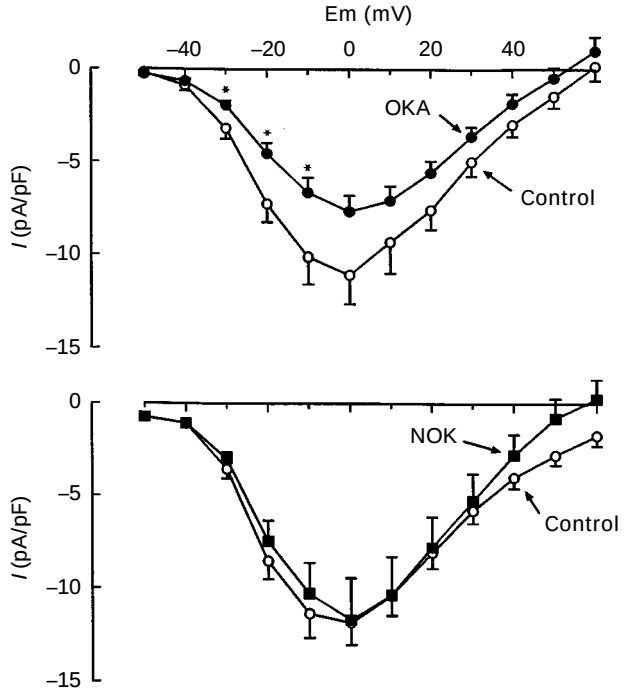

Figure 6 Effects of okadaic acid (OKA) and 1-norokadaone (NOK) on voltage-dependent Ca^{2+} currents in mouse β cells. After isolation the islets were dispersed into single cells which were then cultured for 1–2 days, before being preincubated for 60 min in a medium containing 10 mM glucose and, for test islets, 0.5 μM OKA or 0.5 μM NOK. Ca^{2+} currents were then studied as described in the Methods. Values are means for 11–16 cells from 4 separate cultures; vertical lines show s.e.mean. $*P<0.05$.

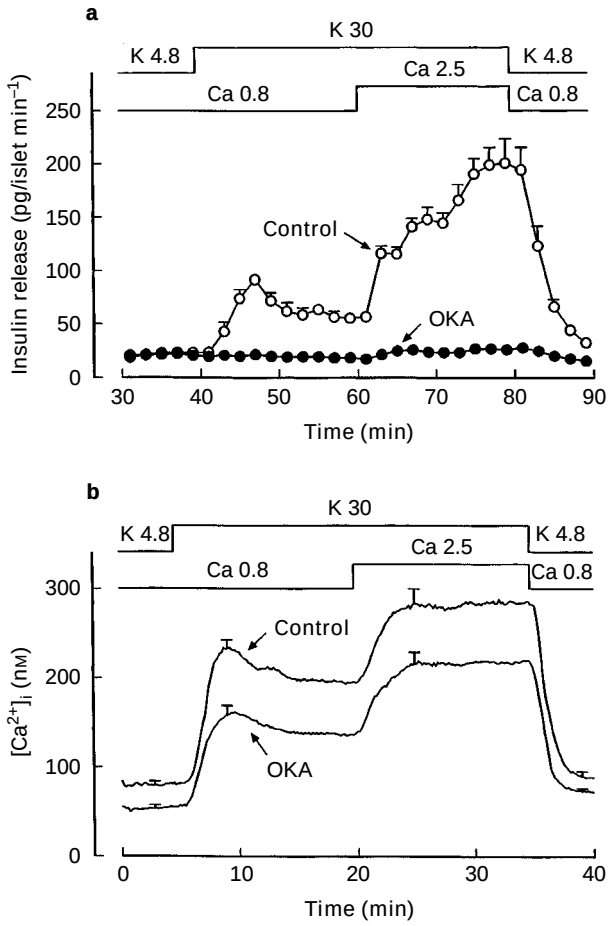

Figure 7 Effects of okadaic acid (OKA) on (a) insulin release and (b) $[Ca^{2+}]_i$ in K^+ stimulated mouse islets. After isolation the islets were cultured for 1 day before being preincubated for 60 min in a medium containing 10 mM glucose without or with 0.5 μM OKA. When $[Ca^{2+}]_i$ was to be measured, the islets were also loaded with fura PE3 for 120 min, OKA being added during the last 60 min of this preincubation period. The measurements of insulin secretion (batches of 20 islets) and $[Ca^{2+}]_i$ (single islets) were performed in different perifusion systems. The perifusion medium contained 15 mM glucose and 250 μM diazoxide throughout, whereas the concentrations of Ca^{2+} and K^+ were changed as indicated. Values are means for 5 experiments of release and for 12 islets from 3 separate cultures for $[Ca^{2+}]_i$; vertical lines show s.e.mean.

phatase activity and protein phosphorylation in intact islets would be necessary to prove it.

In the normal β cell, the response to glucose involves an acceleration of metabolism, an increase in the ATP/ADP ratio, closure of K^+-ATP channels, depolarization of the plasma membrane, activation of voltage-dependent Ca^{2+} channels, influx of Ca^{2+} and a rise in $[Ca^{2+}]_i$ (Ashcroft & Rorsman, 1989; Henquin, 1994). Glucose metabolism, which is exquisitely sensitive to noxious agents, was not impaired in OKA- or CLA-treated islets, as shown by the normal increase in NAD(P)H fluorescence and rise in ATP/ADP ratio. A mere toxic action of the two drugs can thus be excluded. Defects in the closure of K^+-ATP channels and depolarization of the membrane are also unlikely to be involved, because neither direct blockade of the channels with tolbutamide nor depolarization of the membrane with high K^+ in the presence of diazoxide reversed the inhibition of insulin secretion by OKA or CLA.

The first defective step of stimulus-secretion coupling appears to be the influx of Ca^{2+}. Thus, both glucose- and K^+-induced rises in $[Ca^{2+}]_i$ were attenuated in OKA and CLA-treated islets. As Ca^{2+} currents were similarly inhibited after OKA (CLA could not be tested), we suggest that inhibition of PP1 and PP2A eventually impairs Ca^{2+} entry through voltage-dependent Ca^{2+} channels. This is surprising because phosphorylation is generally thought to facilitate Ca^{2+} channel activation (Hescheler *et al.*, 1988; Groschner *et al.*, 1996), including in β cells (Ashcroft & Rorsman, 1989). In agreement with this concept, brief (1–2 min) treatment of mouse β cells with 0.1 μM OKA has been shown to increase Ca^{2+} currents marginally (Ämmälä *et al.*, 1994). However, the same group did not find any acute effects of 1 μM OKA on $[Ca^{2+}]_i$ in intact islets (Zaitsev *et al.*, 1995). It is possible that the difference between these and the present results is explained by the duration of the treatment (short vs long) with OKA. Neither in our experiments (P. Mariot, unpublished results) nor in other studies (Hescheler *et al.*, 1988; Ämmälä *et al.*, 1994) was there any evidence that OKA might be a direct Ca^{2+} channel blocker. It should also be noted that a slight inhibition of Ca^{2+} currents by OKA treatment of intact cells has been observed previously in certain other cell types (Lang *et al.*, 1991; Ward *et al.*, 1991; Groschner *et al.*, 1995).

The 20–25% inhibition of the $[Ca^{2+}]_i$ rise caused by OKA certainly contributes to but cannot entirely explain the almost complete suppression of insulin release. Even when $[Ca^{2+}]_i$ was higher in OKA-treated than control islets (during stimulation with high K^+ and different extracellular Ca^{2+} concentrations), the secretory rate was much lower. Moreover, the peak of insulin release evoked by acetylcholine in the absence of extracellular Ca^{2+} was almost abolished by OKA, although intracellular Ca^{2+} mobilization was only marginally decreased. From these observations we conclude that the efficacy of Ca^{2+} on secretion is decreased after treatment of the islets with OKA.

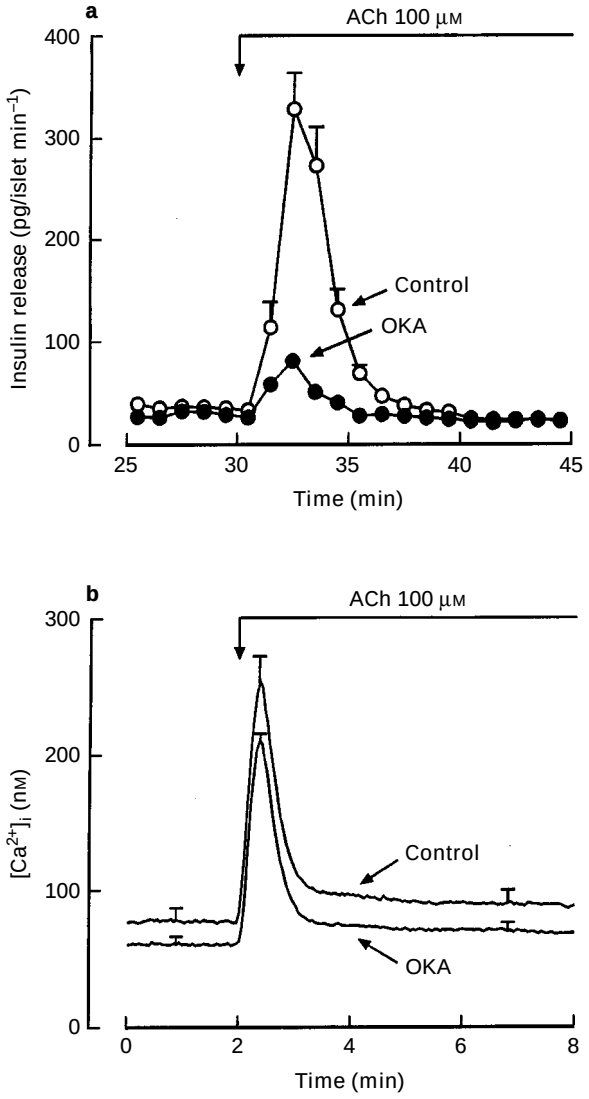

Figure 8 Effects of okadaic acid (OKA) on insulin release and $[Ca^{2+}]_i$ in acetylcholine (ACh)-stimulated mouse islets. After isolation the islets were cultured for 1 day before being preincubated for 60 min in a medium containing 10 mM glucose in the absence or presence of 0.5 μM OKA. When $[Ca^{2+}]_i$ was to be measured, the islets were also loaded with fura PE3 for 120 min, OKA being added during the last 60 min of this preincubation period. The measurements of insulin secretion (batches of 25 islets) and $[Ca^{2+}]_i$ (single islets) were performed in different perifusion systems. The perifusion medium contained 15 mM glucose and was devoid of Ca^{2+}. ACh (100 μM) was added as indicated. Values are means for 6 experiments of release and for 12 islets from 3 separate cultures for $[Ca^{2+}]_i$; vertical lines show s.e.mean.

Two approaches have previously been used to study the effect of OKA on exocytosis of insulin granules. In electrically permeabilized islets, acute application of OKA increased insulin release, but this effect was paradoxical in being observed only in the presence of a non-stimulating concentration of Ca^{2+} (50 nM) while being larger than that of 10 μM free Ca^{2+} (Ratcliff & Jones, 1993). It is unclear whether the proteins whose phosphorylation might be affected by OKA in intact cells are still operative in the model of permeabilized cells. Exocytosis has also been estimated by measuring changes in membrane capacitance in voltage-clamped depolarized β cells. Application of OKA for a few minutes was now found to have no effect on basal exocytosis but to increase the response to Ca^{2+} influx (Ämmälä *et al*., 1994). However, it should be kept in mind that capacitance measurements evaluate exocytosis of large amounts of insulin granules over a few seconds at the most, whereas direct measurements, like those used here, study release of physiological amounts of insulin over many minutes.

Activation of protein kinases A and C potentiates insulin release at least partly by increasing the efficacy of cytoplasmic Ca^{2+} on the secretory machinery (Ämmälä *et al*., 1994; Henquin, 1994; Howell *et al*., 1994). The inhibition of secretion brought about by OKA and CLA could theoretically result from a lesser production of adenosine 3′:5′-cyclic monophosphate (cyclicAMP) or diacylglycerol. This is unlikely because the potentiation of insulin secretion by exogenous cyclicAMP and by a phorbol ester was also impaired by the protein phosphatase inhibitors. OKA exerts a broad spectrum inhibition of insulin secretion that is reminiscent of that of catecholamines, galanin and somatostatin, which act through a pertussis toxin-sensitive G_i-protein (Sharp, 1996). However, pertussis toxin treatment of the islets antagonized the inhibition by adrenaline but did not prevent that by OKA.

Glucose and other nutrients increase the efficacy of Ca^{2+} on insulin secretion (Gembal *et al*., 1992; 1993; Sato *et al*., 1992), but the underlying mechanism is still unknown. It does not depend on activation of protein kinases A or C but is correlated with the ATP/ADP ratio (Gembal *et al*., 1993). The present study suggests that changes in protein phosphatase activity could contribute to this K^+-ATP channel-independent regulation of insulin secretion. Glucose has recently been found to cause a transient increase in PP1-PP2A activity in rat islets (Murphy & Jones, 1996), an effect that could explain the acute increase in acetyl-CoA carboxylase that the sugar produces (Zhang & Kim, 1995). Thus, by preventing dephosphorylation of the enzyme, OKA inhibited its activity in a β cell line (Zhang & Kim, 1995). This may be expected to decrease the level of malonyl-CoA and, therefore, to prevent any rise in cytoplasmic long-chain acyl-CoA esters, which are potential positive modulators of the effect of $[Ca^{2+}]_i$ on insulin secretion (Prentki *et al*., 1992).

In conclusion, OKA inhibits insulin release by decreasing both the magnitude and the efficacy of the Ca^{2+} signal in β cells. We are not aware of other drugs producing similar effects. If one accepts that the effects of OKA are mediated by an inhibition of protein phosphatases, it appears that the concept of a positive regulation of insulin secretion by protein phosphorylation is an oversimplification. The present study indicates that some phosphorylated proteins might exert an inhibitory action in β cells. Dephosphorylation of certain proteins may be as important as phosphorylation of others to promote optimal Ca^{2+} entry and action. Whether these proteins are distal effector proteins (e.g. Ca^{2+} channels, proteins involved in exocytosis) or enzymes of an upstream cascade of phosphorylations/dephosphorylations remains to be established. Activation of protein kinases certainly plays a positive role but activation of certain phosphatases might also be important at certain steps of stimulus-secretion coupling.

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References

ÄMMÄLÄ, C., ELIASSON, L., BOKVIST, K., BERGGREN, P.O., HONKANEN, R.E., SJÖHOLM, Å. & RORSMAN, P. (1994). Activation of protein kinases and inhibition of protein phosphatases play a central role in the regulation of exocytosis in the pancreatic B-cells. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 4343–4347.

AMMON, H.P.T., HEURICH, R.O., KOLB, H.A., LANG, F., SCHAICH, R., DREWS, G. & LEIERS, T. (1996). The phosphatase inhibitor okadaic acid blocks KCl-depolarization-induced rise of cytosolic calcium of rat insulinoma cells (RINm5F). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 95–101.

ASHCROFT, S.J.H. (1980). Glucoreceptor mechanisms and the control of insulin release and biosynthesis. *Diabetologia*, **18,** 5–15.

ASHCROFT, S.J.H. (1994). Protein phosphorylation and beta-cell function. *Diabetologia*, **37,** (Suppl. 2), S21–S29.

ASHCROFT, F.M. & RORSMAN, P. (1989). Electrophysiology of the pancreatic β-cell. *Prog. Biophys. Mol. Biol.*, **54,** 87–143.

ASHIZAWA, N., KOBAYASHI, F., TANAKA, Y. & NAKAYAMA, K. (1989). Relaxing action of okadaic acid, a black sponge toxin on the arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, **162,** 971–976.

BIALOJAN, C. & TAKAI, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.*, **256,** 283–290.

COHEN, P. (1989). The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.*, **58,** 453–508.

DETIMARY, P., JONAS, J.C. & HENQUIN, J.C. (1995). Possible links between glucose-induced changes in the energy state of pancreatic B-cells and insulin release: unmasking by decreasing a stable pool of adenine nucleotides in mouse islets. *J. Clin. Invest.*, **96,** 1738–17453.

GAGLIARDINO, J.J., KRINKS, M.H. & GAGLIARDINO, E.E. (1991). Identification of the calmodulin-regulated protein phosphatase, calcineurin, in rat pancreatic islets. *Biochim. Biophys. Acta*, **1091,** 370–373.

GEMBAL, M., DETIMARY, P., GILON, P., GAO, Z.Y. & HENQUIN, J.C. (1993). Mechanisms by which glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B-cells. *J. Clin. Invest.*, **91,** 871–880.

GEMBAL, M., GILON, P. & HENQUIN, J.C. (1992). Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B-cells. *J. Clin. Invest.*, **89,** 1288–1295.

GROSCHNER, K., SCHUHMANN, K., BAUMGARTNER, W., PASTUSHENKO, V., SCHINDLER, H. & ROMANIN, C. (1995). Basal dephosphorylation controls slow gating of L-type Ca^{2+} channels in human vascular smooth muscle. *FEBS Lett.*, **373,** 30–34.

GROSCHNER, K., SCHUHMANN, K., MIESKES, G., BAUMGARTNER, W. & ROMANIN, C. (1996). A type 2A phosphatase-sensitive phosphorylation site controls modal gating of L-type Ca^{2+} channels in human vascular smooth-muscle cells. *Biochem. J.*, **318,** 513–517.

GILON, P. & HENQUIN, J.C. (1992). Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic B-cell. *J. Biol. Chem.*, **267,** 20713–20720.

HABY, C., LARSSON, O., ISLAM, M.S., AUNIS, D., BERGGREN, P.O. & ZWILLER, J. (1994). Inhibition of serine/threonine protein phosphatases promotes opening of voltage-activated L-type Ca^{2+} channels in insulin secreting cells. *Biochem. J.*, **298,** 341–346.

HARDIE, D.G., HAYSTEAD, T.A.J. & SIM, A.T.R. (1991). Use of okadaic acid to inhibit protein phosphatases in intact cells. *Methods Enzymol.*, **201,** 469–476.

HENQUIN, J.C. (1978). D-glucose inhibits potassium efflux from pancreatic islet cells. *Nature*, **271,** 271–273.

HENQUIN, J.C. (1994). The cell biology of insulin secretion. In *Joslin's Diabetes Mellitus*. ed. Khan, C.R. & Weir, G.C. 13th ed. pp. 56–80. Philadelphia: Lea and Febiger.

HESCHELER, J., MIESKES, G., RÜEGG, J.C., TAKAI, A. & TRAUTWEIN, W. (1988). Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. *Pflügers Arch.*, **412,** 248–252.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.*, **92,** 145–159.

HOWELL, S.L., JONES, P.M. & PERSAUD, S.J. (1994). Regulation of insulin secretion: the role of second messengers. *Diabetologia*, **37,** (Suppl. 2), S30–S35.

ISHIHARA, H., MARTIN, B.L., BRAUTIGAN, D.L., KARAKI, H., OZAKI, H., KATO, Y., FUSETANI, N., WATABE, S., HASHIMOTO, K., UEMURA, D. & HARTSHORNE, D.J. (1989). Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun.*, **159,** 871–877.

LANG, R.J., OZOLINS, I.Z. & PAUL, R.J. (1991). Effects of okadaic acid and ATPγS on cell length and Ca^{2+}-channel currents recorded in single smooth muscle cells of the guinea-pig taenia caeci. *Br. J. Pharmacol.*, **104,** 331–336.

LIANG, Y. & MATSCHINSKY, F.M. (1994). Mechanisms of action of nonglucose insulin secretagogues. *Annu. Rev. Nutr.*, **14,** 59–81.

MAYER, P., JOCHUM, C., SCHATZ, H. & PFEIFFER, A. (1994). Okadaic acid indicates a major function for protein phosphatases in stimulus-response coupling of RINm5F rat insulinoma cells. *Exp. Clin. Ednocrinol.*, **102,** 313–319.

MURPHY, L.I. & JONES, P.M. (1996). Phospho-serine threonine phosphatases in rat islets of Langerhans: identification and effect on insulin secretion. *Mol. Cell. Endocrinol.*, **117,** 195–202.

NISHIWAKI, S., FUJIKI, H., SUGANUMA, M., FURUYA-SUGURI, H., MATSUSHIMA, R., LIDA, Y., OJIKA, M., YAMADA, K., UEMURA, D., YASUMOTO, T., SCHMITZ F. & SUGIMURA, T. (1990). Structure-activity relationship within a series of okadaic acid derivatives. *Carcinogenesis*, **11,** 1837–1841.

PERSAUD, S.J., WHEELER-JONES, C.P.D. & JONES, P.M. (1996). The mitogen-activated protein kinase pathway in rat islets of Langerhans: studies on the regulation of insulin secretion. *Biochem. J.*, **313,** 119–124.

PRENTKI, M., VISCHER, S., GLENNON, M.C., REGAZZI, R., DEENEY, J.T. & CORKEY, B.E. (1992). Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J. Biol. Chem.*, **267,** 5802–5810.

RATCLIFF, H. & JONES, P.M. (1993). Effects of okadaic acid on insulin secretion from rat islets of Langerhans. *Biochim. Biophys. Acta*, **1175,** 188–191.

SATO, Y., AIZAWA, T., KOMATSU, M., OKADA, N. & YAMADA, T. (1992). Dual functional role of membrane depolarization/Ca^{2+} influx in rat pancreatic B-cell. *Diabetes*, **41,** 438–443.

SHARP, G.W.G. (1996). Mechanisms of inhibition of insulin release. *Am. J. Physiol.*, **271,** C1781–C1799.

SHENOLIKAR, S. (1994). Protein serine/threonine phosphatases. New Avenues for cell regulation. *Annu. Rev. Cell. Biol.*, **10,** 55–86.

SJÖHOLM, A., HONKANEN, R.E. & BERGGREN, P.O. (1993). Characterization of serine/threonine protein phosphatases in RINm5F insulinoma cells. *Bioscience Reports*, **13,** 349–358.

STURGESS, N.C., KOZLOWSKI, R.Z., CARRINGTON, C.A., HALES, C.N. & ASHFORD, M.L.J. (1988). Effects of suphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.*, **95,** 83–94.

TAMAGAWA, T., IGUCHI, A., UEMURA, K., MIURA, H., NONOGAKI, K., ISHIGURO, T. & SAKAMOTO, N. (1992). Effects of the protein phosphatase inhibitors okadaic acid and calyculin A on insulin release from rat pancreatic islets. *Endocrinol. Jap.*, **39,** 325–329.

WARD, S.M., VOGALIS, F., BLONDFIELD, D.P., OZAKI, H., FUSETANI, N., UEMURA, D., PUBLICOVER, N.G. & SANDERS, K.M. (1991). Inhibition of electrical slow waves and Ca^{2+} currents of gastric and colonic smooth muscle by phosphatase inhibitors. *Am. J. Physiol.*, **261,** C64–C70.

YANAGIHARA, N., TOYOHIRA, Y., KODA, Y., WADA, A. & IZUMI, F. (1991). Inhibitory effect of okadaic acid on carbachol-evoked secretion of catecholamines in cultured bovine adrenal medullary cells. *Biochem. Biophys. Res. Commun.*, **174,** 77–83.

ZAITSEV, S.V., EFENDIC, S., ARKHAMMAR, P., BERTORELLO, A.M. & BERGGREN, P.O. (1995). Dissociation between changes in cytoplasmic free Ca^{2+} concentration and insulin secretion as evidenced from measurements in mouse single pancreatic islets. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 9712–9716.

ZHANG, S. & KIM, K.H. (1995). Glucose activation of acetyl-CoA carboxylase in association with insulin secretion in a pancreatic β-cell line. *J. Endocrinol.*, **147,** 33–41.

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