Inhibition of serine/threonine protein phosphatases promotes opening of voltage-activated L-type Ca^{2+} channels in insulin-secreting cells

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The biological activity of many proteins, including voltage-sensitive ion channels, is controlled by their state of phosphorylation. Ca^{2+} influx through voltage-activated L-type Ca^{2+} channels serves as the major stimulatory signal in insulin-secreting cells. We have now investigated the extent to which Ca^{2+} handling in clonal insulin-secreting RiNm5F cells was affected by okadaic acid, an inhibitor of various serine/threonine protein phosphatases. Whole-cell patch-clamp experiments showed that okadaic acid generated an increase in membrane current, suggesting that it promotes Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels probably by modifying their phosphorylation state. Okadaic acid was found to provoke a transient rise in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), but had no further effect on the K^+-induced increase. The Ca^{2+} transient induced by okadaic acid was dependent on the presence of extracellular Ca^{2+} and was abolished by D600, a blocker of voltage-activated L-type Ca^{2+} channels. Concomitant with the rise in $[Ca^{2+}]_i$, okadaic acid induced insulin secretion, a phenomenon that was also dependent on extracellular Ca^{2+}. It is proposed that hyperphosphorylation of voltage-activated L-type Ca^{2+} channels in insulin-secreting cells lowers the threshold potential for their activation.

INTRODUCTION

Several reports suggest that the opening probability of voltage-sensitive Ca^{2+} channels is dependent on their state of phosphorylation (for review, see Walaas and Greengard, 1991). The purified L-type Ca^{2+} channel has been found to be subject to phosphorylation by several protein kinases *in vitro*. In the pituitary cell line GH3, these channels maintain their responsiveness through phosphorylation (Armstrong and Eckert, 1987). In the pancreatic β-cell, the voltage-dependent L-type Ca^{2+} channels are directly modulated by cyclic AMP- and protein kinase C-induced phosphorylation, as well as by glucose metabolism (for review, see Ashcroft and Rorsman, 1989).

Insulin release from the β-cell is critically dependent on extracellular Ca^{2+}. Glucose, which is an important insulin secretagogue, is rapidly metabolized in the β-cell, resulting in closure of the ATP-regulated K^+ channels, depolarization, opening of voltage-activated L-type Ca^{2+} channels, and an increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), thereby initiating the secretory process (Arkhammar et al., 1987).

On the other hand, voltage-sensitive Ca^{2+} channels are rapidly inactivated by the Ca^{2+} which enters through the channels during depolarization. This phenomenon may be caused by dephosphorylation of the Ca^{2+} channel, probably through protein phosphatase-2B (calcineurin) activation (Hosey et al., 1986; Kalman et al., 1988). Protein phosphatases-1 or -2A are also candidates for inactivating these Ca^{2+} channels (Hescheler et al., 1987). It is noteworthy, especially for the β-cell, that fructose 2,6-bisphosphate and glucose 1,6-bisphosphate have been shown to serve as non-competitive inhibitors of protein phosphatase-2A (Erickson and Killilea, 1992).

The toxin okadaic acid (OA), produced by the marine plankton dinoflagellates, is a potent and specific inhibitor of protein phosphatases type-1, -2A (Bialojan and Takai, 1988) and -3 (Honkanen et al., 1991), whereas type-2B is only slightly affected, and type-2C is not affected (Bialojan and Takai, 1988). Using OA as a tool, we were interested in elucidating a possible role of protein phosphatases in the regulation of voltage-activated L-type Ca^{2+} channels, $[Ca^{2+}]_i$ and resulting exocytosis in clonal insulin-secreting RiNm5F cells.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and, if not otherwise stated, obtained from Sigma. OA was purchased from LC Services Corp. (Woburn, MA, U.S.A.) and microcystin-LR from Calbiochem. Stock solutions were made up in dimethyl sulphoxide and further diluted in buffer A (see below). The same amount of dimethyl sulphoxide was added for all the controls. Fura-2 acetoxymethyl ester (AM) was from Calbiochem.

Cell culture

RiNm5F cells were cultured in RPMI 1640 medium supplemented with 10% fetal-calf serum, penicillin (100 i.u./ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air.

Electrophysiology

After culture for 24 h, cells were washed with a solution [(mM): choline chloride (138), KCl (5.6), $MgCl_2$ (1.2), $CaCl_2$ (10), tetraethylammonium chloride (10), Hepes (5)] at pH 7.4 and finally placed in 350 μl of the same medium. The pipette solution contained (mM): *N*-methyl-D-glucamine (NMDG) (150), HCl (110), $MgCl_2$ (1), $CaCl_2$ (2), EGTA (10), Mg-ATP (3), Hepes (5), at pH 7.15. All experiments were performed at room temperature (22–24 °C). The free concentration of Ca^{2+} was calculated to be 60 nM from the binding constants of Martell and Smith (1974). NMDG was substituted for K^+ in the pipette solution, in order to block outward-directed K^+ currents. Likewise, choline chloride

Abbreviations used: OA, okadaic acid; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; NMDG, *N*-methyl-D-glucamine; I_{Ca}, Ca^{2+} current.

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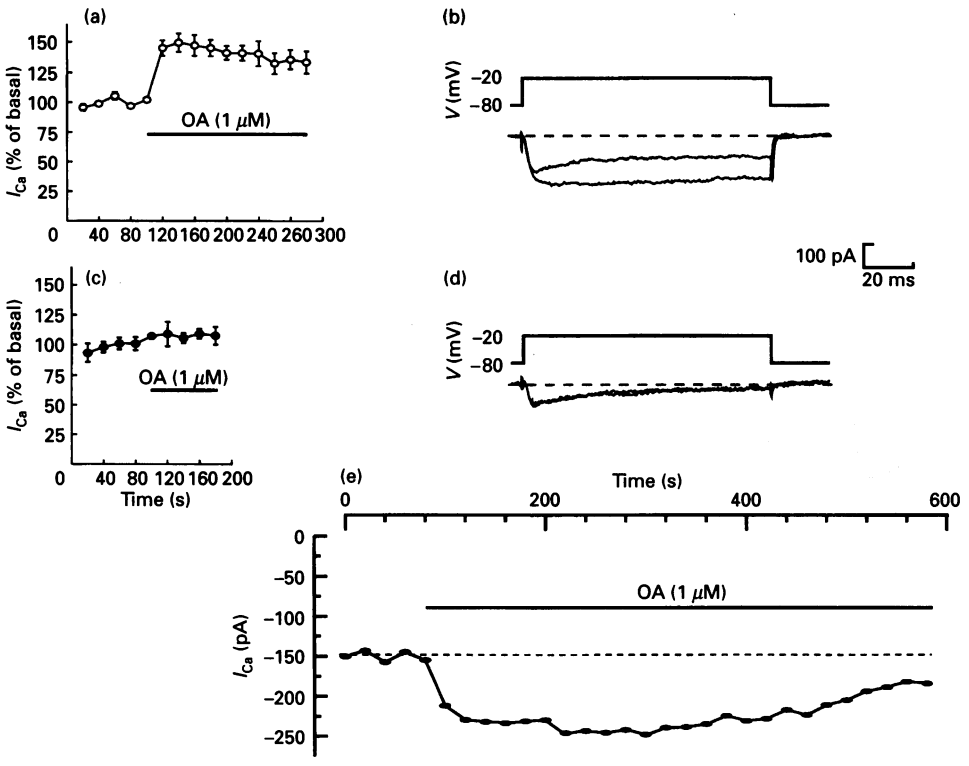

Figure 1 Effect of OA on I_{Ca} in RiNm5F cells

(**a**) Whole-cell currents recorded from cells placed in an extracellular-like buffer. Cells were depolarized (100 ms) to −20 mV from a holding potential of −80 mV every 20 s. After five depolarizing voltage-steps, OA was added (final concn. 1 μM). The five control currents, before addition of OA, were averaged and set to 100%. After addition of OA, the whole-cell I_{Ca} increased by approx. 45% ($P < 0.001$%, $n = 5$). (**b**) Representative whole-cell current traces from the same cell before 20 s after addition of 1 μM OA. The cell was depolarized from −80 mV to −20 mV for 100 ms. Note the effect of OA on the inactivation of the current. (**c**) Effect of 1 μM OA on whole-cell I_{Ca} in the presence of 20 μM Cd^{2+}. Data were obtained and calculated as described in (**a**) ($n = 5$). (**d**) Representative whole-cell Ca^{2+} current traces with and without 1 μM OA obtained from the same cell pretreated with Cd^{2+}. (**e**) Representative I_{Ca} recorded from a cell during exposure to 1 μM OA over a long period of time.

was substituted for NaCl in the extracellular medium, in order to block inward-directed Na^+ currents. The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used, by utilizing an Axopatch 200 patch-clamp amplifier (Axon Instruments, U.S.A.). Voltage-steps were generated, digitized and stored by using the program pClamp (Axon Instruments) and Labmaster ADC (Scientific Solutions, U.S.A.). The current responses were filtered at 1 kHz, Bessel filter (−3 dB-point). The pulse protocol is given in the Figure legend.

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

For $[Ca^{2+}]_i$ measurements, 1-day old cultures grown on coverslips were incubated with 1 μM fura-2/AM in the culture medium for 30 min at 37 °C. After washing, the coverslip was put at the bottom of an open perifusion chamber (volume 150 μl) connected to a two-channel peristaltic pump, allowing constant superfusion of the cells. The chamber was mounted on a thermostatically controlled stage of an inverted fluorescence microscope (Zeiss Axiovert 35M) controlled by a microscope system processor (MSP 21; Zeiss). Fluorescence measurements were performed at 37 °C, with excitation and emission wavelengths of 340/380 and 510 nm respectively. Cells were superfused at a flow rate of 0.5 ml/min with buffer A [(mM): NaCl (125), KCl (5.9), $CaCl_2$ (1.3), $MgCl_2$ (1.2), glucose (3), BSA (1 mg/ml) and Hepes (25)], pH 7.4. The various additions were made in the same buffer. Typically, two to four cells were analysed for $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is expressed as the ratio of the fluorescence measured at 340 nm and 380 nm.

Measurements of Ca^{2+} in permeabilized cells

The cells, washed twice with a cold nominally Ca^{2+}-free buffer [(buffer B) containing (mM): NaCl (10), KCl (110), $MgCl_2$ (1), KH_2PO_4 (2), BSA (0.5 mg/ml) and Hepes (25)] at pH 7.0, were permeabilized by high-voltage electrical discharges. After six pulses of 3.2 kV/cm, more than 95% of the cells became permeabilized, as verified by Trypan Blue uptake. Then 8 μl of concentrated cell suspension was added to a Plexiglass chamber, containing 52 μl of incubation buffer, with continuous stirring. The incubation buffer consisted of buffer B supplemented with 2 mM Mg-ATP, an ATP-regenerating system (10 mM phosphocreatine and 20 units/ml creatine kinase) and mitochondrial inhibitors (0.4 μM antimycin and 2 μg/ml oligomycin). Changes in the ambient free Ca^{2+} concentration were continuously monitored by Ca^{2+}-selective mini-electrodes. All experiments were performed at room temperature. None of the substances used in the study interfered with electrode function.

Measurements of insulin release

Insulin release was studied by perifusing RiNm5F cells mixed with Bio-Gel P-4 polyacrylamide beads (Bio-Rad) in a 0.5 ml column at 37 °C, as previously described (Arkhammar et al.,

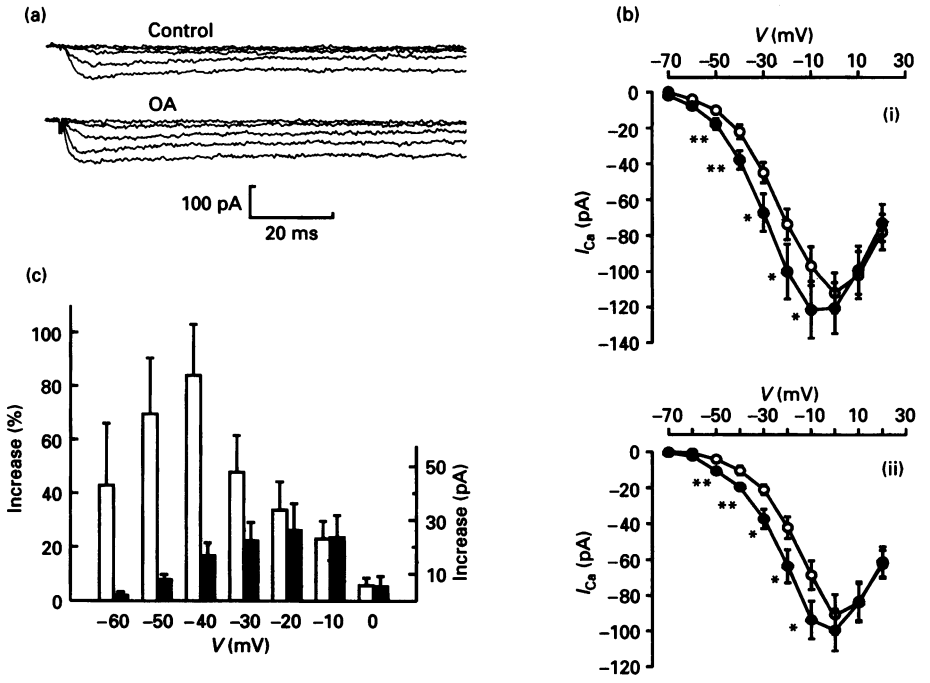

Figure 2 Effect of OA on current–voltage relationship

Membrane currents were recorded during depolarizing voltage steps (100 ms) to membrane potentials between −60 and +20 mV, from a holding potential of −80 mV. At approx. 1 min after establishing the whole-cell configuration, the cells were depolarized in the absence of OA and shortly after addition of 1 μM OA. (**a**) Typical effect of OA on membrane currents. The current traces are from the same cell before and after addition of the phosphatase inhibitor. (**b**) Peak (i) and steady-state (ii) current–voltage relationship before (○) and after (●) addition of 1 μM OA. Data shown as means ± S.E.M. ($n = 8$): $^*P < 0.05$; $^{**}P < 0.01$. (**c**) Compiled data (means ± S.E.M., $n = 8$) showing the effect of OA on membrane currents after depolarizations to membrane potentials as indicated. The absolute increase (black bars) shows currents obtained after subtraction of control currents from the currents obtained in the presence of OA. When expressed as percentage increase over control currents (white bars), the effect is most pronounced at low voltages. Statistical significance was evaluated by Student's t test (paired data).

1987). The flow rate was 0.15 ml/min, and 2 min fractions were collected and analysed for insulin radioimmunologically, with rat insulin as the standard.

RESULTS

OA activates voltage-gated L-type Ca^{2+} channels

Figure 1(a) shows the acute effect of 1 μM OA on whole-cell Ca^{2+} currents in RiNm5F cells. The cells were depolarized to −20 mV from a holding potential of −80 mV. Depolarizing voltage-steps were given every 20 s. On administration of 1 μM OA to the extracellular medium, a stimulation of the peak Ca^{2+} current (I_{Ca}) by approx. 45% ($P < 0.1\%$) was observed. The stimulatory effect was seen in approx. 80% of the cells, and then always induced an increase in the I_{Ca} at the pulse directly after the addition of OA. In several cells, OA not only increased the peak current, but also slowed down the inactivation of the I_{Ca}, as exemplified in Figure 1(b). RiNm5F cells contain both L- and T-type Ca^{2+} channels. We therefore evaluated whether the stimulatory effect of OA was specific to one of the channel types. We found that OA exerted its effect via the L-type Ca^{2+} channels, since 20 μM Cd^{2+}, a blocker of L-type Ca^{2+} channels, totally prevented the stimulatory effect of OA on whole-cell peak currents (Figure 1c) as well as the effect of OA on the inactivation of the I_{Ca} (Figure 1d). The current traces in Figure 1(d) show that the resulting current after addition of Cd^{2+} had fast inactivation properties, typical of those of T-type Ca^{2+} channels. Under these conditions, there was no effect of OA on either peak Ca^{2+} currents or I_{Ca} inactivation. It has recently been reported that the RINm5F cells also contain conotoxin-sensitive 'neuronal' Ca^{2+} channels (Sher et al., 1992). However, the effects of OA on the whole-cell Ca^{2+}-currents reported in the present study cannot be explained by an interaction with these channels; although the conotoxin-sensitive Ca^{2+} channels are sensitive to Cd^{2+}, they typically would have shown inactivation properties similar to those of the T-type Ca^{2+} channels ($\tau \approx 50$–80 ms). In fact, the resulting current after exposure to OA was typical of the L-type Ca^{2+} channel with slow ($\tau > 500$ ms) inactivation properties. Although the effect of OA decreased slightly with time, the stimulatory effect was maintained for at least 10 min in the continued presence of OA (Figure 1e).

In another set of experiments, we investigated the effect of OA on the current–voltage relationship in the insulin-secreting cell line (Figure 2). I_{Ca} was evoked in 8 cells by depolarizing voltage steps from −60 to +20 mV, from a holding potential of −80 mV. The absolute increase in I_{Ca} was obtained at voltages around −20 mV. However, the relative increase (expressed as percentage increase over control) was most pronounced at lower voltages. In most of the cells, I_{Ca} could be evoked at lower voltages than before the addition of OA. This indicates that OA promotes influx of Ca^{2+} through voltage-dependent L-type channels, by affecting their voltage-dependency.

OA promotes an increase in $[Ca^{2+}]_i$

Figure 3(a) shows the acute effect of 1 μM OA on $[Ca^{2+}]_i$ measured in a small cluster of RiNm5F cells. Addition of 1 μM OA to the perifusion medium for about 1 min instantaneously provoked a transient rise in $[Ca^{2+}]_i$. This occurred whether or not

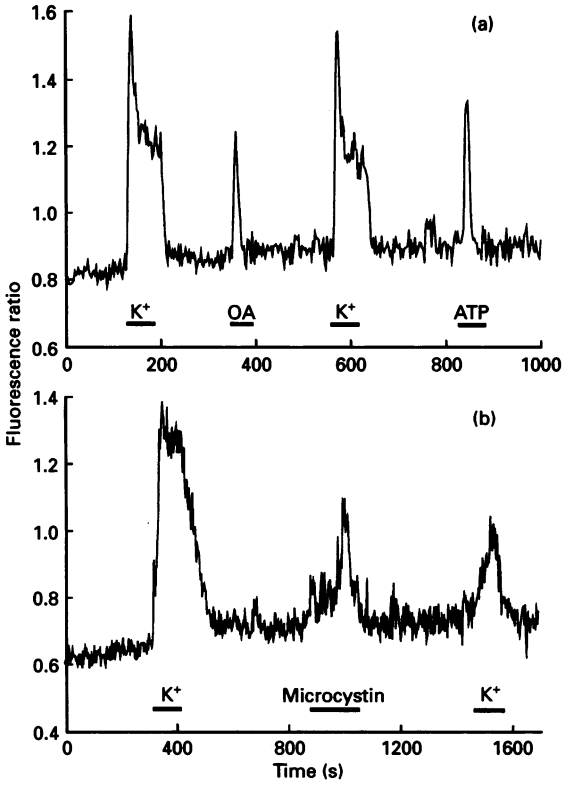

Figure 3 Effect of OA on $[Ca^{2+}]_i$

RiNm5F cells were perifused with buffer A as described in the Materials and methods section. Additions of 25 mM KCl or 200 μM ATP were as indicated; 1 μM OA (**a**) or 1 μM microcystin-LR (**b**) was added, and changes in $[Ca^{2+}]_i$ were measured in clusters of 2–4 cells. The traces shown are representative of 10 different experiments for OA and 3 for microcystin-LR.

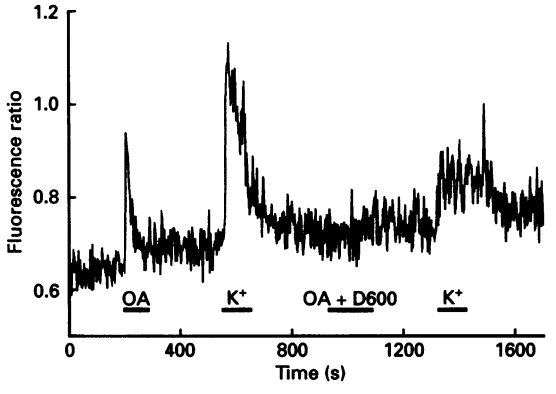

Figure 4 Effect of the Ca^{2+}-channel blocker D600 on OA-induced increase in $[Ca^{2+}]_i$

OA (1 μM) and D-600 (50 μM) were added as indicated. $[Ca^{2+}]_i$ measurements were carried out and expressed as described in the Materials and methods section. The trace shown is representative of 3 different experiments.

OA addition was preceded by Ca^{2+} entry provoked by K^+-induced depolarization (see also Figure 4). OA had no noticeable further effect on the $[Ca^{2+}]_i$ increase evoked by K^+ stimulation,

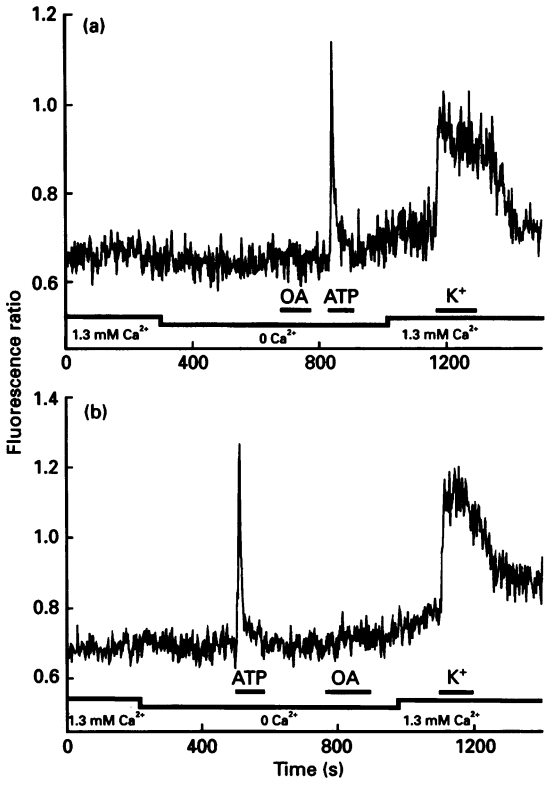

Figure 5 Effect of extracellular Ca^{2+} on the OA- and ATP-induced increase in $[Ca^{2+}]_i$

RINm5F cells were incubated with buffer A (1.3 mM Ca^{2+}) or with Ca^{2+}-deprived buffer A (0 Ca^{2+}); 1 μM OA was added before (**a**) or after (**b**) 200 μM ATP. $[Ca^{2+}]_i$ measurements were carried out and expressed as described in the Materials and methods section. The traces shown are representative of 5 different experiments.

nor on the $[Ca^{2+}]_i$ increase induced by 200 μM ATP. The effect of ATP reflects Ins(1,4,5)P_3-induced mobilization of intracellularly stored Ca^{2+}, after stimulation of the purinergic P2 receptor (Arkhammar et al., 1990). When cells were perifused with 1 μM microcystin-LR, another potent and specific inhibitor of serine/threonine protein phosphatases (Honkanen et al., 1990) (Figure 3b), there was a rise in $[Ca^{2+}]_i$ similar to that obtained in the presence of OA. It is noteworthy that the OA-induced increase in $[Ca^{2+}]_i$ reversed, although the phosphatase inhibitor was still present (Figure 3a), which seems to contradict the patch-clamp experiments, where the effect of OA was maintained for a prolonged period of time (Figure 1). However, it should be kept in mind that some experimental conditions (cell integrity and temperature) are different. To what extent such differences fully explain the discrepancy merits further investigations.

The $[Ca^{2+}]_i$ rise is abolished by D600

Figure 4 shows that the OA-induced Ca^{2+} transient was totally abolished by the concomitant addition of 50 μM D600, a blocker of voltage-dependent L-type Ca^{2+} channels. Combined with the results from the patch-clamp experiments presented in Figure 2, the data suggest that OA promotes phosphorylation of the voltage-gated L-type Ca^{2+}-channel protein and thereby changes its threshold for activation, resulting in Ca^{2+} influx at lower membrane potentials.

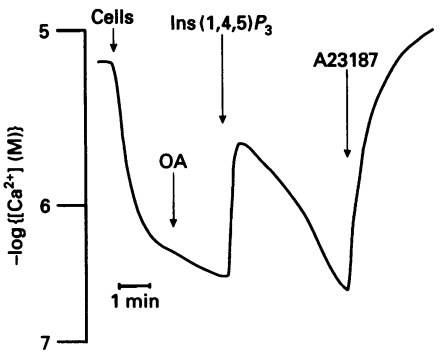

Figure 6 Effect of OA on the Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilized RiNm5F cells

Electropermeabilized RiNm5F cells (3.9×10^7 cells/ml) were incubated in an intracellular-like buffer containing ATP, an ATP-regenerating system and mitochondrial inhibitors. Changes in the ambient free Ca^{2+} concentration were measured by Ca^{2+}-sensitive mini-electrodes. OA (1 μM) was added 2 min before addition of 5 μM Ins(1,4,5)P_3, as indicated by the arrows. To check the overall ability of the intracellular Ca^{2+} stores to release Ca^{2+}, the Ca^{2+} ionophore A23187 (4 μM) was added at the end of the experiment. The traces shown are representative of 4 different experiments.

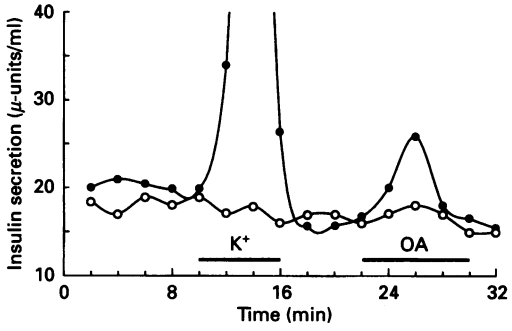

Figure 7 Effect of OA on the dynamics of insulin release

RiNm5F-cell suspensions were perifused as described in the Materials and methods section with buffer A (●) or with Ca^{2+}-deprived buffer A (○). Additions of 25 mM KCl or 1 μM OA were made as indicated. The maximal insulin release induced by KCl was 78 μ-units/ml. The curves are representative of 4 different experiments.

The $[Ca^{2+}]_i$ rise is dependent on extracellular Ca^{2+}

The transient rise in $[Ca^{2+}]_i$, induced by OA, was dependent on extracellular Ca^{2+}. Figure 5(a) shows that removal of extracellular Ca^{2+} from the perifusion medium abolished the rise in $[Ca^{2+}]_i$ elicited by OA, without affecting the Ca^{2+} response induced by a subsequent addition of ATP. Similar results were obtained when ATP was applied before the addition of OA (Figure 5b), demonstrating that the OA-induced $[Ca^{2+}]_i$ transient was independent of intracellular Ca^{2+} mobilization. When Ca^{2+}-containing buffer was added back, cells regained their responsiveness to K^+-induced depolarization. These results further support the concept that OA promotes Ca^{2+} entry into the cells. It is further strengthened by data presented in Figure 6. In electropermeabilized RiNm5F cells OA had no effect on Ca^{2+} uptake, nor on Ins(1,4,5)P_3-induced Ca^{2+} release (Figure 6). Moreover, OA did not affect the overall Ca^{2+} content of the intracellular pools, as evident from the effect of the Ca^{2+} ionophore A23187. Also when cells were treated with OA before permeabilization, there was no effect of the phosphatase inhibitor on Ins(1,4,5)P_3-induced Ca^{2+} release (results not shown). These data show that the intracellular Ca^{2+} pools are not disturbed by suppression of the actual OA-sensitive protein phosphatase activities.

OA promotes insulin release

RiNm5F cells are well known for secreting insulin, an effect mediated by an increase in $[Ca^{2+}]_i$. It can therefore be expected, in view of our results, that OA by promoting Ca^{2+} entry into the cells would also promote insulin secretion. Figure 7 shows that addition of 1 μM OA indeed produced about 50% increase in insulin secretion over basal, an effect considerably smaller than that triggered by K^+-induced depolarization. Both OA-induced and K^+-induced insulin secretion were abolished by removing Ca^{2+} from the extracellular milieu, a condition which also abolished their effect on $[Ca^{2+}]_i$ (see Figure 5).

DISCUSSION

Protein phosphorylation/dephosphorylation processes are known to be of crucial importance in controlling a vast array of cellular functions. The present study describes the involvement of protein phosphorylation in the regulation of voltage-gated L-type Ca^{2+}-channel activity in insulin-secreting cells. It is demonstrated that the serine/threonine protein phosphatase inhibitors OA and microcystin-LR elicited Ca^{2+} entry into clonal insulin-producing RiNm5F cells. This effect was not modified by a previous transient $[Ca^{2+}]_i$ increase, provoked by K^+-induced depolarization. The fact that microcystin-LR had similar effects to those of OA in our experimental system suggests that this compound somehow had sufficient access to the phosphatase to inhibit it. Whole-cell patch-clamp experiments showed that OA affected the activation of voltage-gated L-type Ca^{2+} channels. This observation indicates that OA, by suppressing dephosphorylation directly of these Ca^{2+} channels or of proteins closely associated with them, enhances their state of phosphorylation. This leads to an increased opening probability of the voltage-gated Ca^{2+} channels. One must assume, however, that the protein phosphatase(s) inhibited by OA and the protein kinase(s) responsible for phosphorylation of the channel express basal activity under non-stimulatory conditions. This implies that it is actually the imbalance in phosphorylation, provoked by OA, that is responsible for the increased Ca^{2+} current. It should be emphasized that we have no experimental data supporting the notion that the observed effect of the phosphatase inhibitors on $[Ca^{2+}]_i$ could be accounted for by membrane depolarization promoted by a direct interaction with the ATP-regulated K^+ channels (O. Larsson and P.-.O. Berggren, unpublished work).

It is well documented that phosphorylation increases the opening probability of voltage-activated Ca^{2+} channels (for review, see Walaas and Greengard, 1991). In cardiac myocytes, Hescheler et al. (1988) reported that OA increases the Ca^{2+} current 2-fold in cells superfused with threshold concentrations of isoprenaline, whereas the membrane currents were only slightly affected by 1–5 μM OA in the absence of the agonist. Interestingly, a high concentration (50 μM) of OA did increase the Ca^{2+} current even in the absence of adrenergic stimulation. Similarly, in bovine chromaffin cells, recruitment of facilitation Ca^{2+} current by voltage is normally reversible, but OA renders it irreversible (Artalejo et al., 1992). From the present $[Ca^{2+}]_i$ measurements, it is obvious that OA (1 μM) acts without prior stimulation or major depolarization, which may be explained by

a distinct availability or an elevated basal activity of the enzyme(s) responsible for the phosphorylation of the channel. Direct measurements of membrane potential (O. Larsson and P.-O. Berggren, unpublished work) clearly show that the RiNm5F cells are not fully at rest under the present experimental conditions. Hence the OA-induced changes in threshold potential for activation of the Ca^{2+} channel, as observed in the whole-cell patch-clamp experiments, may well explain the effect of the phosphatase inhibitor on $[Ca^{2+}]_i$.

The mechanisms of insulin secretion in response to glucose have been extensively explored. The increase in $[Ca^{2+}]_i$ serves as the major trigger signal for insulin release (Arkhammar et al., 1987). We found that OA, by promoting Ca^{2+} entry into the cells, also induced insulin secretion. It appears that insulin secretion represents one physiological response, among the array of events triggered by OA-induced Ca^{2+} influx. Addition of OA has been shown to increase basal noradrenaline secretion in the absence of Ca^{2+} in digitonin-permeabilized chromaffin cells (Wu and Wagner, 1991), but not in digitonin-permeabilized PC12 cells (Wagner and Vu, 1990), without affecting the amount of noradrenaline secreted in the presence of Ca^{2+}. On the other hand, OA has been reported to inhibit carbachol-evoked secretion of catecholamines in adrenal cells (Yanagihara et al., 1991). These experiments clearly show that events other than modulation of Ca^{2+}-channel activity participate in OA-induced secretion.

OA is a potent inhibitor of several protein phosphatases *in vitro*, among them protein phosphatases type-1, -2A (Bialojan and Takai, 1988) and -3 (Honkanen et al., 1991), IC_{50} being 10, 0.1 and 5 nM respectively. Type-2B is inhibited with a much lower potency ($IC_{50} = 5\ \mu M$), and it is therefore unlikely that this protein phosphatase is involved in the regulation of the processes discussed here. Since the ability of OA to inhibit the various protein phosphatases is critically dependent on their actual concentrations, the toxin can only be used as a tool to discriminate between them in cell-free assays (for review, see Cohen, 1991). Because the phosphatases are present in cells at high concentrations, 1 μM OA is required to block them totally in intact cells, and selective inhibition of protein phosphatases-1, -2A or -3 is not possible (Hardie et al., 1991). More selective inhibitors are needed in order to characterize the protein phosphatase inhibited by OA in the present study. Nevertheless, it is shown that protein phosphatase activity is critical in maintaining the phosphorylation state, and hence the activity, of voltage-gated L-type Ca^{2+} channels. Hyperphosphorylated Ca^{2+} channels, elicited by OA, are activated at more negative membrane potentials than for those possessing a normal state of phosphorylation. Our results lead to the hypothesis that the phosphatase activity, which controls phosphorylation of the channel protein, may be essential in determining the threshold potential for activation of the voltage-gated L-type Ca^{2+} channel in insulin-secreting cells.

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REFERENCES

Arkhammar, P., Nilsson, T., Rorsman, P. and Berggren, P. O. (1987) J. Biol. Chem. **262**, 5448–5454
Arkhammar, P., Hallberg, A., Kindmark, H., Nilsson, T., Rorsman, P. and Berggren, P. O. (1990) Biochem. J. **265**, 203–211
Armstrong, D. and Eckert, R. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 2518–2522
Artalejo, C. R., Rossie, S., Perlman, R. L. and Fox, A. P. (1992) Nature (London) **358**, 63–66
Ashcroft, F. M. and Rorsman, P. (1989) Prog. Biophys. Mol. Biol. **54**, 87–143
Bialojan, C. and Takai, A. (1988) Biochem. J. **256**, 283–290
Cohen, P. (1991) Methods Enzymol. **201**, 389–399
Erickson, A. K. and Killilea, S. D. (1992) Biochem. Int. **27**, 353–359
Hamill, O. P., Marty, A., Neher, E., Sackman, B. and Sigworth, F. J. (1981) Pflügers Arch. **391**, 85–100
Hardie, D. G., Haystead, T. A. J. and Sim, A. T. R. (1991) Methods Enzymol. **201**, 469–481
Hescheler, J., Kameyama, M., Trautwein, W., Mieskes, G. and Söling, H. D. (1987) Eur. J. Biochem. **165**, 261–266
Hescheler, J., Mieskes, G., Rüegg, J. C., Takai, A. and Trautwein, W. (1988) Pflügers Arch. **412**, 248–252
Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L., Khatra, B. S., Dukelow, M. and Boynton, A. L. (1990) J. Biol. Chem. **265**, 19401–19404
Honkanen, R. E., Zwiller, J., Daily, S. L., Khatra, B. S., Dukelow, M. and Boynton, A. L. (1991) J. Biol. Chem. **266**, 6614–6619
Hosey, M. M., Borsotto, M. and Lazdunski, M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 3733–3737
Kalman, D., O'Lague, P. H., Erxleben, C. and Armstrong, D. L. (1988) J. Gen. Physiol. **92**, 531–548
Martell, A. E. and Smith, R. M. (1974) Critical Stability Constants, Vol. I: Amino Acids; Vol. II: Amines, Plenum Press, New York
Sher, E., Biancardi, E., Pollo, A., Carbone, E., Li, G., Wollheim, C. B. and Clementi, F. (1992) Eur. J. Pharmacol. **216**, 407–414
Wagner, P. D. and Vu, N. D. (1990) J. Biol. Chem. **265**, 10352–10357
Walaas, S. I. and Greengard, P. (1991) Pharmacol. Rev. **43**, 299–349
Wu, Y. N. and Wagner, P. D. (1991) Biochim. Biophys. Acta **1092**, 384–390
Yanagihara, N., Toyohira, Y., Koda, Y., Wada, A. and Izumi, F. (1991) Biochem. Biophys. Res. Commun. **174**, 77–83

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