Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4343–4347, May 1994
Cell Biology

Activation of protein kinases and inhibition of protein phosphatases play a central role in the regulation of exocytosis in mouse pancreatic β cells

(insulin/membrane capacitance/secretion/pancreas/Ca^{2+})

CARINA ÄMMÄLÄ*, LENA ELIASSON*, KRISTER BOKVIST*, PER-OLOF BERGGREN†, RICHARD E. HONKANEN‡, ÅKE SJÖHOLM†, AND PATRIK RORSMAN*§

*Department of Medical Biophysics, Medicinaregatan 11, S-413 90 Göteborg, Sweden; †Rolf Luft Centre for Diabetes Research, Department of Endocrinology, Karolinska Institute, Box 60500, S-171 76 Stockholm, Sweden; and ‡Department of Biochemistry, University of South Alabama, Mobile, AL 36688

Communicated by Rolf Luft, January 24, 1994 (received for review September 3, 1993)

ABSTRACT **The mechanisms that regulate insulin secretion were investigated using capacitance measurements of exocytosis in single β cells maintained in tissue culture. Exocytosis was stimulated by voltage-clamp depolarizations to activate the voltage-dependent Ca^{2+} channels that mediate Ca^{2+} influx into the β cell. Under basal conditions, the exocytotic responses were small despite large Ca^{2+} currents. The exocytotic responses were dramatically increased (10- to 20-fold) by conditions that promote protein phosphorylation, such as activation of protein kinases A and C or inhibition of protein phosphatases. The stimulation of secretion was not due to an enhancement of Ca^{2+} influx and both peak and integrated Ca^{2+} currents were largely unaffected. Our data indicate that exocytosis in the insulin-secreting pancreatic β cell is determined by a balance between protein phosphorylation and dephosphorylation. They further suggest that although Ca^{2+} is required for the initiation of exocytosis, modulation of exocytosis by protein kinases and phosphatases, at a step distal to the elevation of Ca^{2+}, is of much greater quantitative importance. Thus an elevation of Ca^{2+} may represent a permissive rather than a decisive factor in the regulation of the insulin secretory process.**

Knowledge about the molecular processes that control exocytosis in the insulin-secreting pancreatic β cell remains fragmentary. Earlier experiments have suggested that an elevation of the cytoplasmic Ca^{2+} concentration triggers insulin release (1), possibly via activation of Ca^{2+}-dependent protein kinases (2). In addition, exocytosis is modulated by hormones and neurotransmitters, many of which act by activation of protein kinases (3–5). Here we have explored the influence of protein phosphorylation on the voltage-dependent L-type Ca^{2+} currents and insulin secretion in single metabolically intact voltage-clamped pancreatic β cells by using the patch-clamp technique and high-resolution capacitance measurements of exocytosis (6). By using voltage-clamped cells, it is possible to exclude the possibility that the observed actions on secretion are secondary to changes in membrane potential with resultant effects on the frequency of the Ca^{2+}-dependent action potentials (2). Our data suggest that insulin secretion is critically dependent on the phosphorylation of certain, as yet unidentified, regulatory proteins that modulate exocytosis at a level distal to the elevation of Ca^{2+}.

MATERIALS AND METHODS

Preparation of Cells. Normal mouse β cells were used throughout the study. The mice were stunned by a blow against the head and killed by cervical dislocation and decapitation. The pancreas was quickly removed and pancreatic islets were isolated by collagenase digestion. Single cells were prepared as described (7), plated on Corning tissue culture dishes, and maintained in tissue culture for up to 3 days in RPMI 1640 tissue culture medium, containing 5 mM glucose and supplemented with streptomycin (100 μg/ml) and penicillin (100 international units/ml).

Electrophysiology. Whole-cell membrane currents were recorded from individual β cells by using the perforated-patch whole-cell configuration as described (3, 8). Perforation required a few minutes and the voltage clamp was regarded as satisfactory when the series conductance exceeded 40 nS. The holding potential was −70 mV and depolarizations were 500 ms long and went to 0 mV. Exocytosis was measured as changes in cell capacitance (C_m) as described elsewhere (9). The interval between each measurement of C_m was 100 ms. Depolarizations were applied at low frequency (0.3–0.5 min^{-1}). It was ascertained that stable capacitance responses were obtained before addition of a compound so that the observed changes are not just long-term trends. Effects on the Ca^{2+} current were assessed by measuring both the peak amplitude of the Ca^{2+} current (I_{Ca}) and the integrated Ca^{2+} current ([$Q_{Ca} = \int I_{Ca}(t)dt$]).

Solutions. The extracellular medium consisted of 118 mM NaCl, 20 mM tetraethylammonium chloride, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 5 mM Hepes (pH 7.4 with NaOH). Glucose was included in the extracellular solution at a concentration of 5 mM to provide the metabolic fuel required to energize the secretory process. However, subsequent experiments have indicated that secretion proceeds well in the complete absence of the sugar. The pipette solution contained 76 mM Cs_2SO_4, 10 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes·CsOH (pH 7.35). Electrical contact was established by the addition of amphotericin B (Sigma) to the pipette solution. Briefly, a stock solution containing 6 mg of amphotericin dissolved in 100 μl of dimethyl sulfoxide was prepared. Twenty microliters of this stock solution was then added to 5 ml of the pipette solution, yielding a final concentration of 0.24 mg/ml. The tip of the pipette was filled with amphotericin-free solution and the pipette was then back-filled with amphotericin-containing solution. Okadaic acid (Molecular Probes), the phorbol ester phorbol 12-myristate 13-acetate (PMA), and forskolin (both from Sigma) were dissolved in dimethyl sulfoxide (final concentration of dimethyl sulfoxide, 0.02–0.1%). In Figs. 1 and 4, the cells were pretreated with forskolin for >15 min. This protocol was used as it was difficult to maintain the recording long enough to permit multiple additions and to

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; PKC and PKA, protein kinases C and A, respectively.

§To whom reprint requests should be addressed.

allow a new steady-state level to be attained before the subsequent additions of either PMA or okadaic acid. All experiments were performed at 32–34°C.

Immunoblot Analysis. Immunoblot analysis on Immobilon-P was carried out essentially as described elsewhere (10). Two identical aliquots of the purified catalytic subunits of PP1 and PP2A were subjected to SDS/PAGE on a 10% polyacrylamide gel alongside of a whole β cell homogenate. The gel was transferred to Immobilon-P electrophoretically using a Trans-Blot system (Bio-Rad). Gels were transferred at 50 V per h per cm, and staining of the gels after transfer indicated the transfer was essentially complete. The blot was cut in two and incubated for 1 h in phosphate-buffered saline containing 5% (wt/vol) nonfat dried milk to reduce nonspecific binding. The blot was then incubated with polyclonal antibodies produced against a synthetic peptide corresponding to the C-terminal amino acids of PP1 (anti-PP1) or PP2A (anti-PP2A). Immunoreactivity of the primary antibody was visualized with commercial chemiluminescence system (ECL–Amersham), using biotin-labeled goat anti-rabbit antibodies as described by the manufacturer.

Data Analysis. Data are presented as the mean ± SEM and statistical significances are evaluated using Student's *t* test.

RESULTS

As shown in Fig. 1*A*, activation of protein kinase C (PKC) by inclusion of the phorbol ester PMA (10 nM) in the extracellular solution had only a small effect on the voltage-clamp Ca^{2+} current and yet dramatically enhanced the exocytotic response. On average (Fig. 1*B*), PMA increased the peak Ca^{2+} current by 35 ± 10%, the integrated current by 30 ± 10%, and the exocytotic response by 320 ± 60%. The effects on exocytosis are consistent with previously reported actions of this phorbol ester on insulin secretion (11).

The effects of PKC activation are qualitatively and quantitatively similar to those of protein kinase A (PKA) (4). We therefore explored the interaction between PKC and PKA. Fig. 1*C* shows the effects of PMA (10 nM) on the Ca^{2+} current and exocytosis in a cell already exposed to forskolin, an activator of adenylate cyclase that in itself stimulates exocytosis >4-fold (3). Interestingly, activation of PKC was equally effective as a stimulator of exocytosis under these experimental conditions. On average (Fig. 1*D*), PMA was without effect on the peak and integrated Ca^{2+} current but nevertheless enhanced the exocytotic response by 240 ± 40% over that observed with forskolin alone.

An ≈100% increase in the integrated Ca^{2+} current has previously been demonstrated to double the exocytotic response (3). The observation that PMA maximally increases the Ca^{2+} current by 30%, therefore, indicates that stimulation of Ca^{2+} influx is not the main mechanism by which PMA potentiates insulin secretion. The relatively weak effect of PMA on the Ca^{2+} current is also consistent with the failure

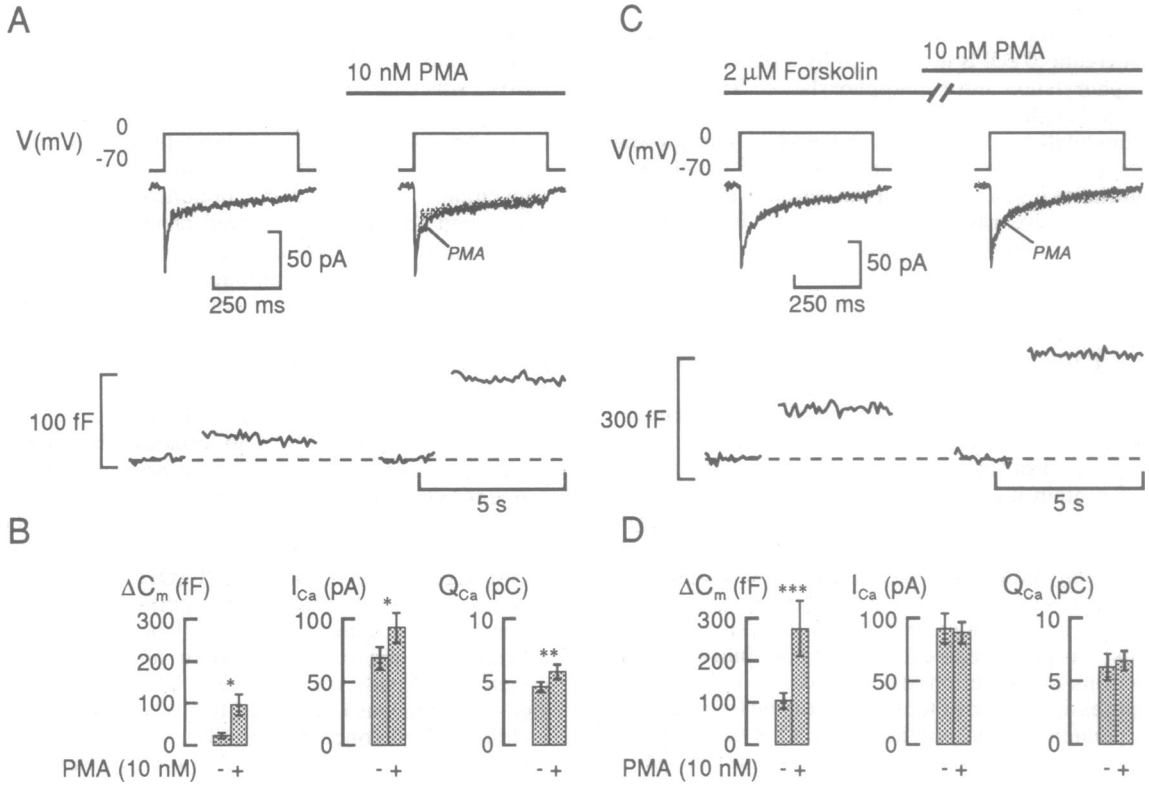

FIG. 1. (*A*) Ca^{2+} currents (upper traces) and changes in cell capacitance (lower traces) evoked by 500-ms depolarizations before and 4 min after addition of 10 nM PMA. Under basal conditions, the peak inward current was 56 pA, the integrated current was 5.6 pC, and the capacitance increase was only 20 fF. After inclusion of PMA in the perfusion medium, the corresponding values were 51 pA, 5.8 pC, and 100 fF, respectively. (*B*) Average effects of PMA on the peak Ca^{2+} current (I_{Ca}), the integrated Ca^{2+} current ($Q_{Ca} = \int I_{Ca}dt$), and the increase in cell capacitance (ΔC_m). (*C*) Ca^{2+} currents and changes in cell capacitance before and 4 min after addition of 10 nM PMA in a cell pretreated for >15 min with 2 μM forskolin. In the presence of forskolin alone, the peak and integrated Ca^{2+} current evoked by a 500-ms depolarization to 0 mV amounted to 90 pA and 11.3 pC, respectively, producing an exocytotic response of 124 fF. After addition of PMA, the corresponding values were 89 pA, 9.5 pC, and 270 fF, respectively. (*D*) Average effects of PMA in the presence of forskolin on the peak Ca^{2+} current (I_{Ca}), the integrated Ca^{2+} current (Q_{Ca}), and the increase in cell capacitance (ΔC_m). (*A* and *C*) The Ca^{2+} currents observed under control conditions or in the presence of forskolin alone are superimposed on those recorded in the presence of PMA to aid comparison of the current responses. The dashed horizontal lines superimposed on the capacitance traces indicate the prestimulatory level. Values are the mean ± SEM of 9–12 experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of this compound to affect glucose-induced β-cell electrical activity (12, 13).

The finding that PMA potentiates exocytosis in excess of that produced by a high concentration of forskolin may indicate that PKC and PKA stimulate insulin release by phosphorylation of distinct proteins. To further explore the role of protein phosphorylation in the control of secretion, we next investigated the effects of okadaic acid (100 nM), a potent inhibitor of type 1, type 2A, and type 3 serine/threonine protein phosphatases (14). Interestingly, okadaic acid enhanced depolarization-evoked exocytosis even when applied in the absence of kinase stimulators (Fig. 2). Okadaic acid stimulated exocytosis by 250 ± 90%. This effect was associated with a slight increase in the peak (+10 ± 5%) and integrated Ca^{2+} current (+40 ± 10%). Again, the increase in the Ca^{2+} current is too small to account for more than a fraction of the effect on exocytosis (see argument above).

As seen in Fig. 3, both PP1 and PP2A were identified by Western blot analysis of mouse β-cell homogenates. These phosphatases represent the major divalent-cation-independent okadaic acid-sensitive protein phosphatases found in most (if not all) animals and tissues examined to date (14, 15). Thus, it is likely that the effects of okadaic acid on the Ca^{2+} current and exocytosis in the β cells are mediated by either of these enzymes.

We next compared the effects of okadaic acid when either PKC or PKA was already activated. Fig. 4*A* shows a recording from a cell pretreated with forskolin for >15 min in which

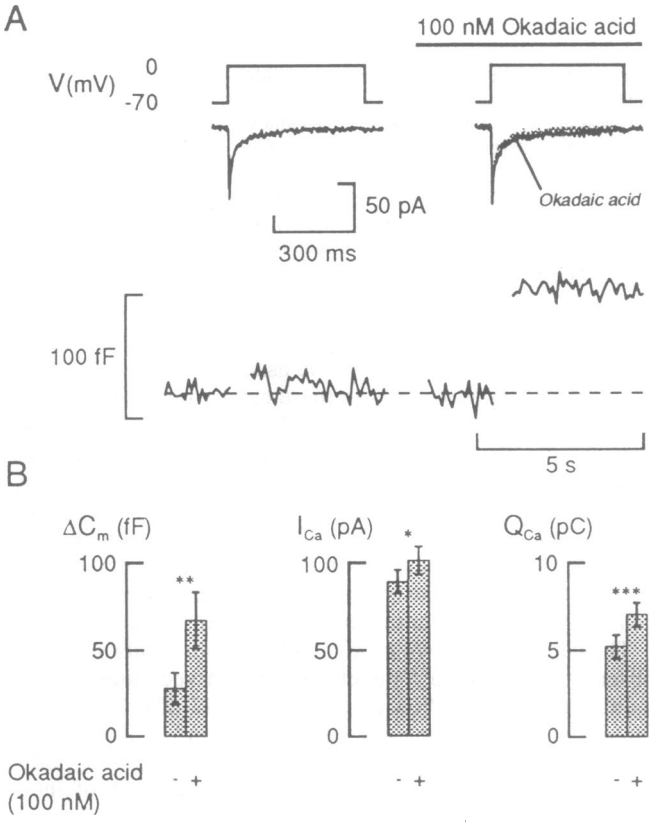

FIG. 2. (*A*) Ca^{2+} currents (upper traces) and changes in cell capacitance (lower traces) evoked by 500-ms depolarizations before and 2 min after addition of 100 nM okadaic acid. Under control conditions, the peak Ca^{2+} current was 74 pA, the integrated current was 3.3 pC, and the associated exocytotic response was only 16 fF. After addition of okadaic acid, the corresponding values were 80 pA, 5.1 pC, and 85 fF, respectively. (*B*) Average effects of okadaic acid on the peak Ca^{2+} current (I_{Ca}), the integrated Ca^{2+} current (Q_{Ca}), and the increase in cell capacitance (ΔC_m). Values are the mean ± SEM of five or six experiments. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$.

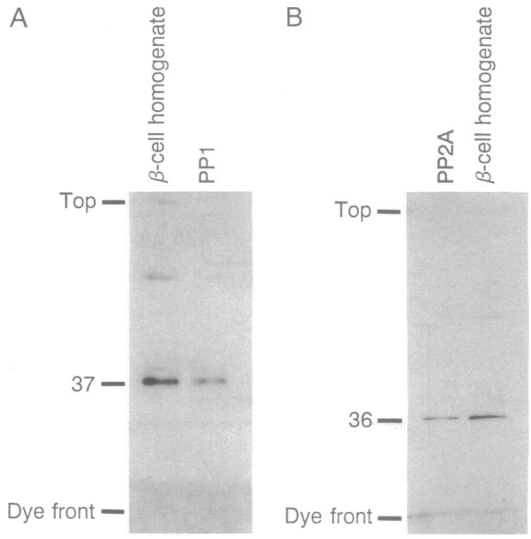

FIG. 3. Immunocytochemical analysis of a β-cell homogenate identifying the presence of PP1 and PP2A. The catalytic subunits of purified PP1 and PP2A from rabbit muscle and a whole β-cell homogenate were analyzed by immunolabeling using polyclonal antibodies specific for the catalytic subunits of PP1 and PP2A. The immunoblots were obtained with a polyclonal antibody specific for PP1 (*A*) and an antibody specific for PP2A (*B*). The top of the gel, the dye front, and molecular masses of 36 and 37 kDa are indicated.

okadaic acid dramatically enhanced exocytosis. On average, the exocytotic response was potentiated by 200 ± 65% over that seen in the presence of forskolin alone. The effect on secretion was associated with an increased Ca^{2+} current; peak Ca^{2+} current rose by 20 ± 5%, and the integrated current increased by 100 ± 50% (Fig. 4*B*). In contrast, okadaic acid had no or only small effects on the Ca^{2+} currents and exocytotic response in cells preincubated with PMA (Fig. 4 *C* and *D*). The weak effect of okadaic acid on PMA-stimulated exocytosis may be attributable to the fact that both activation of PKC and inhibition of the okadaic acid-sensitive phosphatases promote phosphorylation of serine/threonine amino acid residues.

DISCUSSION

We have used measurements of cell capacitance as an indicator of insulin secretion. The properties of exocytosis in the β cell as determined by this technique are in general agreement with those established using measurements of insulin release (2, 3, 9). The large advantage of cell capacitance measurements, as an indicator of the exocytotic rate, over more traditional techniques, measuring insulin release, is that it enables recordings of secretion in individual cells with a high temporal resolution. In this study we have used measurements of cell capacitance to study the role of protein phosphorylation in the control of Ca^{2+}-dependent exocytosis in the insulin-secreting β cell.

Our data suggest that in the pancreatic β cell, as has been proposed for neurotransmitter release in neurons (16), exocytosis is regulated by a dynamic balance between protein phosphorylation and dephosphorylation. Protein phosphorylation appears to result in an increased availability of releasable secretory granules. Whether this is merely the consequence of a chemical modification of the secretory granules and/or docking sites in the plasma membrane or does in fact reflect the actual translocation of the granules within the cells to a location in closer proximity to the release sites remains to be established.

In general, exocytosis is favored by any condition that shifts the balance toward protein phosphorylation—i.e., ei-

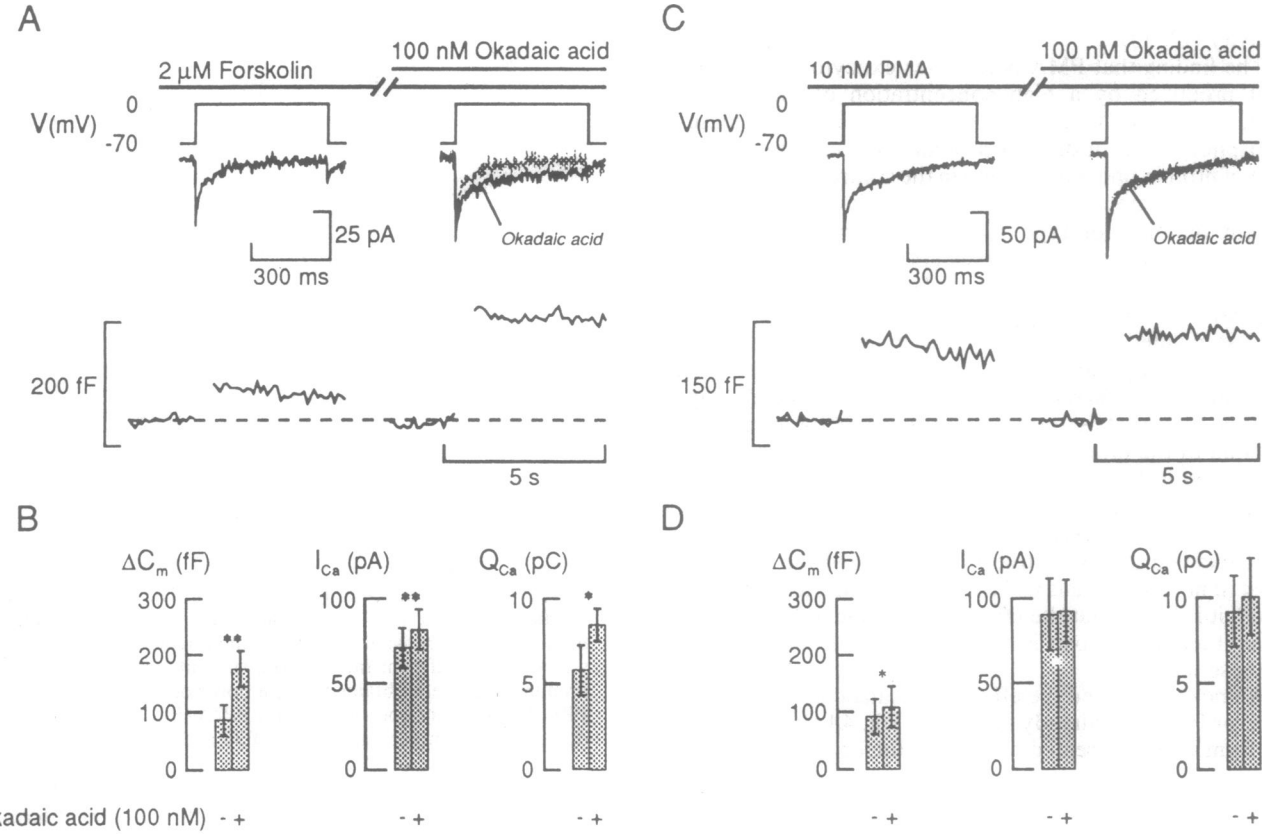

FIG. 4. (*A*) Ca^{2+} currents (top traces) and changes in cell capacitance (lower traces) before and 4 min after addition of okadaic acid (100 nM) in the presence of 2 μM forskolin. In the presence of forskolin alone, the peak Ca^{2+} current was 37 pA, the integrated Ca^{2+} current was 2.2 pC, and the associated capacitance increase was 53 fF. After addition of okadaic acid, the corresponding values had increased to 48 pA, 6.9 pC, and 234 fF, respectively. (*B*) Average effects of okadaic acid on the peak Ca^{2+} current (I_{Ca}), the integrated Ca^{2+} current (Q_{Ca}), and the increase in cell capacitance (ΔC_m) in cells pretreated with forskolin. (*C*) Lack of effect of okadaic acid on Ca^{2+} currents and changes in cell capacitance in β cells pretreated for >15 min with PMA. Records obtained 4 min after addition of the phosphatase inhibitor. In the presence of PMA alone, the peak Ca^{2+} current was 90 pA, the integrated current was 10.7 pC, and the associated exocytotic response was 97 fF. After addition of okadaic acid, the corresponding values were 109 pA, 11.2 pC, and 102 fF, respectively. (*D*) Average effects of okadaic acid on the peak Ca^{2+} current (I_{Ca}), the integrated Ca^{2+} current (Q_{Ca}), and the increase in cell capacitance elicited by 500 ms depolarizations to 0 mV. Values are the mean ± SEM of five to nine experiments. *, $P < 0.05$; **, $P < 0.01$.

ther activation of the kinases or inhibition of the phosphatases. It appears that some activity of the kinases and phosphatases is already present under basal conditions. This is suggested by the observation that the phosphatase inhibitor okadaic acid potentiated exocytosis even when it was applied alone (Fig. 2). Okadaic acid has previously been suggested to enhance synaptic transmission in the neuromuscular junction by increasing neurotransmitter release (17, 18), an effect that was proposed to result from stimulation of Ca^{2+} influx. Considering its weak effect on the integrated Ca^{2+} current in the pancreatic β cell, this does not appear to be the main mechanism by which this phosphatase inhibitor potentiates exocytosis of the insulin-containing secretory granules. Our data are rather consistent with an effect exerted at steps distal to the elevation of Ca^{2+}. It should be pointed out that our finding that okadaic acid stimulates depolarization-induced exocytosis in single β cells (as revealed by the capacitance measurements) is at variance with the recent observation that okadaic acid inhibits insulin secretion from intact pancreatic islets (19). However, this latter effect may be proximal to any direct effect on the exocytotic machinery. For example, interference with glucose uptake and/or metabolism (20) will have profound effects on β-cell electrical activity, transmembrane Ca^{2+} fluxes, and ultimately insulin secretion (2). Consistent with this explanation, okadaic acid was indeed found to stimulate insulin release when applied to permeabilized pancreatic islets (19). In the latter preparation, the influence of such early steps in the sequence of events that culminate in the discharge of insulin can be assumed to be negligible.

Regulation of exocytosis in the β cell by both kinases and phosphatases clearly adds considerable flexibility to the molecular control of the release process. It may turn out that modulation of the activity of protein phosphatases is as important a regulator of cellular processes, such as exocytosis, as is the activity of protein kinases. Accordingly, it is possible that activation of protein phosphatases, by analogy with what has previously been demonstrated in chromaffin cells (21), can result in inhibition of insulin secretion.

The data obtained with PMA and forskolin confirm earlier observations that Ca^{2+}-dependent insulin secretion can be modulated by both PKA and PKC (2–4, 11–13). Given that the effects of the kinases are additive and that they are differently affected by okadaic acid, it is tempting to hypothesize that phosphorylation of a different regulatory protein(s) is involved. It must be emphasized, however, that biochemical experiments of protein phosphorylation in permeabilized islets have failed to demonstrate any unique substrate(s) of PKC (4). Recently, a 145-kDa cytoplasmic protein that is essential for Ca^{2+}-dependent secretion was identified in a number of neuroendocrine cells, including insulin-secreting RINm5F cells (22). With regard to the action of PKC on exocytosis in the β cells, it is of considerable interest that the 145-kDa protein is a PKC substrate and that its presence is required for stimulation of secretion by PKC (23).

Earlier experiments on intact pancreatic islets have led to the suggestion that an elevation of the cytoplasmic Ca^{2+} concentration is a powerful initiator of insulin secretion (1, 2). However, in this type of experiment, it has not been possible to unequivocally discriminate between the action of Ca^{2+} itself and that resulting from the combined effects of Ca^{2+} and the tonic activation of protein kinases (or inhibition of protein phosphatases) due to the exposure of the β cell to a mixture of islet hormones and neurotransmitters released from neighboring endocrine cells as well as intraislet nerve endings (5). In the present study, isolated β cells had to be used to ascertain adequate voltage-clamp, which is a prerequisite for the measurements of cell capacitance. This experimental protocol has the inherent advantage that it precludes any influence of endogenous islet modulators of protein phosphorylation. The present data suggest that an elevation of Ca^{2+} *per se* is a fairly weak initiator of insulin secretion (see controls in Figs. 1 and 2). For a large exocytotic response, either activation of PKA and PKC or inhibition of protein phosphatases is required. Under such experimental conditions, secretion may increase 10- to 20-fold over that seen under conditions of minimal protein phosphorylation without much concomitant stimulation of the Ca^{2+} current. Thus, although Ca^{2+} is certainly required for the initiation of exocytosis, its role appears permissive rather than decisive and the number of insulin-containing granules actually released is determined by the degree of phosphorylation of certain key regulatory proteins. The concept that the secretory response of the β cell is critically dependent on protein phosphorylation is finally consistent with the reported poor secretory capacity of purified β cells and that it can be restored by pharmacological agents that, for example, elevate the cytoplasmic concentrations of cAMP (24).

C.Ä. and L.E. contributed equally to this study and their names appear in alphabetical order. This work was supported by the Juvenile Diabetes Foundation International, the Swedish Medical Research Council, the Åke Wibergs Stiftelse, the Magn. Bergvalls Stiftelse, the Swedish Diabetes Association, the Nordic Insulin Foundation Committee, the Bank of Sweden Tercentenary Foundation, and the Swedish Society for Medicine.

1. Prentki, M. & Matschinsky, F. M. (1987) *Physiol. Rev.* **67,** 1185–1248.
2. Ashcroft, F. M. & Ashcroft, S. J. H. (1992) in *Insulin: Molecular Biology to Pathology*, eds. Ashcroft, F. M. & Ashcroft, S. J. H. (IRL, Oxford), pp. 97–150.
3. Ämmälä, C., Ashcroft, F. M. & Rorsman, P. (1993) *Nature (London)* **363,** 356–358.
4. Jones, P. M., Salmon, D. M. W. & Howell, S. L. (1988) *Biochem. J.* **254,** 397–403.
5. Rasmussen, H., Zawalich, K. C., Ganesan, S., Calle, R. & Zawalich, W. S. (1990) *Diabetes Care* **13,** 655–666.
6. Neher, E. & Marty, A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6712–6716.
7. Rorsman, P. & Trube, G. (1986) *J. Physiol. (London)* **374,** 531–550.
8. Horn, R. & Marty, A. (1988) *J. Gen. Physiol.* **92,** 145–159.
9. Ämmälä, C., Eliasson, L., Bokvist, K., Larsson, O., Ashcroft, F. M. & Rorsman, P. (1993) *J. Physiol. (London)* **472,** 665–688.
10. Honkanen, R. E., Zwiller, J., Daily, S. L., Khatra, B. S., Dukelow, M. & Boynton, A. L. (1991) *J. Biol. Chem.* **266,** 6614–6619.
11. Arkhammar, P., Nilsson, T., Welsh, M., Welsh, N. & Berggren, P.-O. (1989) *Biochem. J.* **264,** 207–215.
12. Pace, C. S. & Goldsmith, K. T. (1985) *Am. J. Physiol.* **298,** C527–C534.
13. Bozem, M., Nenquin, M. & Henquin, J. C. (1987) *Endocrinology* **121,** 1025–1033.
14. Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15,** 98–102.
15. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508.
16. Greengard, P., Valtorta, F., Czernik, A. J. & Benfenati, F. (1993) *Science* **259,** 780–785.
17. Abdual-Ghani, M., Kravitz, E. A., Meiri, H. & Rahamimoff, R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1803–1807.
18. Swain, J. E., Robitaille, R., Dass, G. R. & Charlton, M. P. (1991) *J. Neurobiol.* **22,** 855–864.
19. Ratcliff, H. & Jones, P. M. (1993) *Biochim. Biophys. Acta* **1175,** 188–191.
20. Tanti, J. F., Grémeaux, T., Van Obberghen, E. & Le Marchand-Brustel, Y. (1991) *J. Biol. Chem.* **266,** 2099–2103.
21. Galindo, E., Zwiller, J., Bader, M.-F. & Aunis, D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7398–7402.
22. Walent, J. H., Porter, B. W. & Martin, T. F. (1992) *Cell* **70,** 765–775.
23. Nishizaki, T., Walent, J. H., Kowalchyk, J. A. & Martin, T. F. J. (1992) *J. Biol. Chem.* **267,** 23972–23981.
24. Pipeleers, D., In't Veld, P. A., Maes, E. & van de Winkel, M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7322–7325.