Protein Kinase Activation Increases Insulin Secretion by Sensitizing the Secretory Machinery to Ca^{2+}

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ABSTRACT Glucose and other secretagogues are thought to activate a variety of protein kinases. This study was designed to unravel the sites of action of protein kinase A (PKA) and protein kinase C (PKC) in modulating insulin secretion. By using high time resolution measurements of membrane capacitance and flash photolysis of caged Ca^{2+}, we characterize three kinetically different pools of vesicles in rat pancreatic β-cells, namely, a highly calcium-sensitive pool (HCSP), a readily releasable pool (RRP), and a reserve pool. The size of the HCSP is ~20 fF under resting conditions, but is dramatically increased by application of either phorbol esters or forskolin. Phorbol esters and forskolin also increase the size of RRP to a lesser extent. The augmenting effect of phorbol esters or forskolin is blocked by various PKC or PKA inhibitors, indicating the involvement of these kinases. The effects of PKC and PKA on the size of the HCSP are not additive, suggesting a convergent mechanism. Using a protocol where membrane depolarization is combined with photorelease of Ca^{2+}, we find that the HCSP is a distinct population of vesicles from those colocalized with Ca^{2+} channels. We propose that PKA and PKC promote insulin secretion by increasing the number of vesicles that are highly sensitive to Ca^{2+}.

KEY WORDS: exocytosis • insulin • calcium sensitivity • PKA • PKC

INTRODUCTION

Insulin secretion is subject to precise regulation by nutrient and nonnutrient secretagogues. Despite the well-known depolarization–secretion coupling initiated by metabolism of glucose and other nutrient secretagogues, nutrients also activate intracellular signaling pathways that lead to the activation of protein kinases such as PKC and PKA (Nesher et al., 2002). On the other hand, nutrient-induced insulin responses can be radically modified by nonnutrient secretagogues, including a wide variety of hormones and neurotransmitters, through the same intracellular regulators as nutrient secretagogues. For example, cholinergic muscarinic agonists generate diacylgycerol (DAG) and subsequently activate PKC (Verspohl and Wienecke, 1998), glucagon and glucose-dependent insulinotropic polypeptide elevate cAMP with subsequent activation of PKA, whereas somatostatin, galanin, or the α2-adrenoreceptor agonists inhibit adenylate cyclase and reduce intracellular cAMP (Sharp, 1996).

Although protein kinases have been implicated in the control of insulin secretion, precisely how they participate in producing a controlled insulin response is not well understood. In addition to modulating cell excitability, calcium influx, and gene expression (Bozem et al., 1987; Liu and Heckman, 1998), recent evidence also suggests that PKA or PKC act directly on secretory machinery. In pancreatic β-cells, PKA activation potentiates insulin secretion by increasing the total number of vesicles that are available for release (Renstrom et al., 1997; Rorsman et al., 2000). PKC activation has also been linked to priming of Ca^{2+}-mediated insulin secretion as well as enhancement of non-Ca^{2+}–mediated exocytosis (Eliasson et al., 1996; Efanov et al., 1997). Direct interactions of PKA or PKC with the secretory machinery has also been suggested in other cell types, such as chromaffin cells and hippocampal neurons, where the size of the RRP and its rate of replenishment is increased (Smith et al., 1998; Stevens and Sullivan, 1998). Recently, a direct modulation of the Ca^{2+} sensitivity of fusion by PKC has been demonstrated in chromaffin cells and gonadotropes (Yang et al., 2002; Zhu et al., 2002).

This study investigates the mechanisms whereby insulin secretion is regulated by PKA and PKC in rat pancreatic β-cells. We focused on the secretory response distal to Ca^{2+} signaling by using whole cell membrane capacitance (C_m) measurements and direct and spatially uniform manipulation of $[Ca^{2+}]_i$ with caged Ca^{2+}. We

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Abbreviations used in this paper: DAG, diacylgycerol; HCSP, highly calcium-sensitive pool; IRP, immediately releasable pool; RRP, readily releasable pool.

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have identified a small, highly calcium-sensitive pool (HCSP) in addition to the previously reported RRP and the reserve pool. The size of the HCSP dramatically increased after treatment with PMA or forskolin, which also increased the size of the RRP, albeit to a lesser extent. To better characterize the specific isoforms of PKC involved in the modulation of secretory vesicles, we tested the effects of various PKC inhibitors on secretory responses. Furthermore, we evaluated whether the actions of PKC and PKA converge to influence the secretory process.

MATERIALS AND METHODS

Cell Culture and Solutions

All experiments were performed on isolated rat pancreatic β-cells at 30–32°C. Pancreatic β-cells from adult male Wistar rats were prepared as described previously (Ashcroft et al., 1984). In brief, rats were killed by cervical dislocation, and the islets were collected from the pancreas after collagenase digestion. The islets were further digested by dispase II to dissociate single β-cells. Dispersed cells were kept in DMEM supplemented with 25 mM HEPES, 2 g/L $NaHCO_3$, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cells cultured for 3–5 d were used in the experiments.

Standard bath solution for the experiments contained (in mM) 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 3 glucose, 5 HEPES (pH 7.2, 310 mosm). For pipette solutions, we generally prepared 2× concentrated buffers, which contained 250 mM Cs-glutamate and 80 mM HEPES (pH 7.2). We added to the 2× buffer $CaCl_2$, ATP, GTP, caged Ca^{2+}, and Ca^{2+} indicators. Standard internal solution consisted of (in mM) 110 Cs-glutamate, 2 MgATP, 0.3 GTP, and 35 HEPES with different loaded caged Ca^{2+} of either NP-EGTA or DM-nitrophen, and 0.2 mM various Ca^{2+} indicators, such as fura-6F or furaptra. The basal $[Ca^{2+}]_i$ was measured to be around 200 nM by fura-2. For experiments with depolarization, the bath solution contained 10 mM $CaCl_2$.The pipette solution was adjusted to pH 7.2 with either HCl or CsOH. The osmolarity was adjusted to around 300 mosm.

Stock solutions of forskolin, PMA, Gö6976, and Gö6983 were prepared in DMSO. Stock solutions of PKC19-31 were made in 5% acetic acid. The final concentration of DMSO or acetic acid in diluted solutions was <0.02%. DMEM, Dispase-II, FBS, and BSA were from GIBCO BRL; PKC19-31, Gö6976, and Gö6983 were purchased from Calbiochem; NP-EGTA, DM-nitrophen, fura-2, fura-6F, and furaptra were from Molecular Probes; forskolin, Rp-cAMP, PMA, and all other chemicals were purchased from Sigma-Aldrich.

Membrane Capacitance (C_m) Measurement

We selected cells with diameters >11 μm for study, so that >90% of the cells were expected to be β-cells (Rorsman and Trube, 1986). Conventional whole-cell recordings were conducted using Sylgard-coated pipettes with series resistance ranging from 4 to 12 MΩ. An EPC-9 patch-clamp amplifier was used together with PULSE+LOCK-IN software (Heka Elektronics). A 1042-Hz, 20-mV peak-to-peak sinusoidal voltage stimulus was superimposed on a holding potential of −70 mV. Currents were filtered at 2.9 kHz and sampled at 15 kHz. The capacitance traces were imported to IGOR Pro (WaveMetrics) for further analysis.

Flash Photolysis

Flashes of ultraviolet light and fluorescence–excitation light were generated as described previously (Xu et al., 1997). In the flash experiments, exocytosis was elicited by photorelease of caged Ca^{2+} preloaded into the cell via the patch pipette. Flashes of UV light were generated by a flash lamp (Rapp Optoelektronik). $[Ca^{2+}]_i$ was measured with the Ca^{2+} indicator dyes fura-2, fura-6F, or furaptra. The dyes were excited with light alternating between 340 and 385 nm from a monochromator-based system (TILL photonics). The resulting fluorescence signal was measured by a photomultiplier. $[Ca^{2+}]_i$ was determined from the ratio (R) of the fluorescence signals excited at the two wavelengths, following the equation $[Ca^{2+}]_i = Keff * (R - Rmin)/(Rmax - R)$, where Keff, Rmin, and Rmax are constants obtained from intracellular calibration as previously described (Xu et al., 1997). In brief, four solutions with $[Ca^{2+}]_i$ of nominal zero (10 mM EGTA with no added Ca^{2+}), 20 μM (20 mM DPTA with 4 mM $CaCl_2$), 80 μM (20 mM DPTA with 10 mM $CaCl_2$), and 10 mM (10 mM $CaCl_2$ with no added buffer) were dialyzed against the cytosol in the whole-cell patch clamp recording. Three to five recordings were made for each calibration solution to estimate the calibration constants.

Data Analysis

Data analysis was performed using IGOR Pro software (Wavemetrics), and results were presented as mean ± SEM with the indicated number of experiments. Statistical significance was evaluated using Student's *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics of Secretory Response in Rat Pancreatic β-cells

We first characterized the secretory response to different $[Ca^{2+}]_i$ levels in single rat pancreatic β-cells. Exocytosis was elicited by flash photorelease of Ca^{2+} in the whole-cell patch-clamp configuration. Following a flash, $[Ca^{2+}]_i$ was uniformly elevated to the μM range within few milliseconds. Thus, the sizes of distinct vesicle pools and their secretory kinetics at a given $[Ca^{2+}]_i$ could be studied directly without the complications of $[Ca^{2+}]_i$ microdomains or modulation of Ca^{2+} influx.

Fig. 1 A displays a typical C_m response to a step-like $[Ca^{2+}]_i$ elevation. The C_m trace clearly displayed multiple kinetic components of exocytosis, indicating the presence of different vesicle pools as has been suggested for other cell types (Neher, 1993; Heinemann et al., 1994; Xu et al., 1998; Voets, 2000). Each exponential component of the C_m trace is usually interpreted as release of a discrete vesicle pool, whereas the sustained linear increase is thought to reflect refilling from a reserve pool of vesicles (Sorensen et al., 2002). When we looked into the detailed kinetics of the initial exocytotic burst at an expanded time scale (Fig. 1 B), we observed a small but very fast component of exocytosis at low μM $[Ca^{2+}]_i$. The amplitude of this fast component reflects release of 6–12 vesicles (~20 fF) if we assume that one insulin-containing granule contributes 1.7–3.4 fF of membrane as determined in pancreatic β-cells (Ammala et al., 1993; Braun et al., 2004). This component was readily identifiable in ~50% of the cells (n = 93) studied. This variability likely results from the rela-

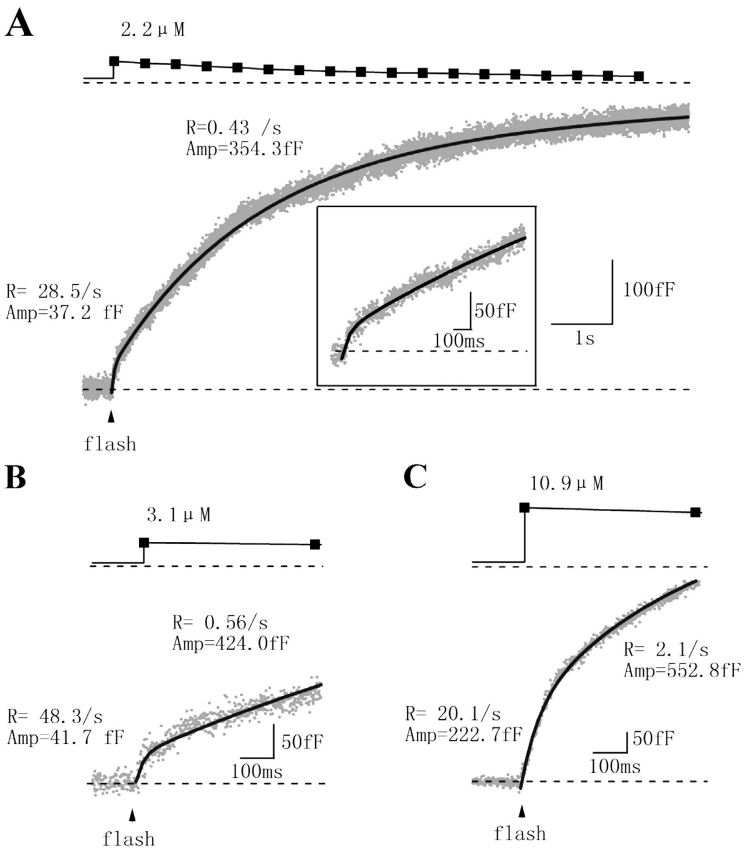

FIGURE 1. The kinetics of exocytosis in pancreatic β-cells at different $[Ca^{2+}]_i$ levels. (A) Example trace of a secretory response following flash photolysis that exhibited two distinct phases. Superimposed is the double exponential fit (solid line) with the rate constants and amplitudes indicated. The exocytotic burst is expanded in the inset. (B and C) Expanded exocytotic bursts in response to different post-flash $[Ca^{2+}]_i$ levels with superimposed double exponential fits (solid lines). (B) At lower $[Ca^{2+}]_i$ levels, a small-amplitude exponential component with a relatively fast rate constant was followed by a slower, but larger amplitude second exponential component. (C) At higher $[Ca^{2+}]_i$ levels, the slower but larger phase of exocytosis dominated the exocytotic burst.

tively small size of this pool and considerable cell-to-cell variation of secretory competence. The most rapid component of exocytosis had a relatively fast time constant of ~20 ms at a $[Ca^{2+}]_i$ level of 3.1 μM. In contrast, recent studies in β-cells report exocytosis from a readily releasable pool (RRP) with an amplitude of ~200 fF and time constants of 1 s or longer upon photoelevation of $[Ca^{2+}]_i$ to ~3 μM (Takahashi et al., 1997; Barg et al., 2001). Thus, this small, fast component in the exocytotic burst at low $[Ca^{2+}]_i$ is kinetically distinguishable from the previously described RRP but is similar to what has recently been described as a highly Ca^{2+}-sensitive pool (HCSP) in chromaffin cells (Yang et al., 2002) and rat insulinoma INS-1 cells (Yang and Gillis, 2004). As we elevated $[Ca^{2+}]_i$ to higher values, a slower but larger phase of exocytosis became dominant (Fig. 1 C). This slower phase had an amplitude (~200 fF) and kinetics comparable to the previously reported RRP (Takahashi et al., 1997; Barg et al., 2001; Olofsson et al., 2002).

PMA Significantly Increased the Size of the HCSP

Next, we tested the effect of PMA, a PKC activator, on exocytosis in pancreatic β-cells. A prominent HCSP component with much larger size than in control cells is evident after PMA treatment (Fig. 2, A and B). When the averaged time courses of C_m increase in response to similar post-flash $[Ca^{2+}]_i$ levels were compared, PMA clearly increased the burst component of exocytosis as well as the sustained component (Fig. 2 C). To investigate the Ca^{2+} dependence of secretion, the exocytotic bursts were further fitted by a double exponential to obtain the rate constants and amplitudes of the HCSP and RRP. Fig. 3 compared the sizes and the rate constants of the HCSP and RRP from control ($n = 93$) and PMA-treated ($n = 117$) cells between $[Ca^{2+}]_i$ levels of 0.7 and 30 μM. Despite the comparable rate constants, the amplitudes of the HCSP were much greater in PMA-treated cells, as shown in the upper part of Fig. 3. We did not observe a clear dependence of the amplitude on $[Ca^{2+}]_i$.

The rate constant of release from the HCSP was dependent on $[Ca^{2+}]_i$ between 0.8 and ~3 μM and saturated at $[Ca^{2+}]_i$ above 3 μM. We fitted the HCSP rate constant to the equation $Rate = R_{max}/(1 + (K_d/[Ca^{2+}]_i)^n)$, and obtained an estimated K_d of 2.5 ± 0.5 μM. The best-fit Hill coefficient (n) was 1.9, suggesting a less cooperativity in the Ca^{2+}-dependent fusion of HCSP compared with the RRP. A shallow dependence of the rate of exocytosis of the HCSP on $[Ca^{2+}]_i$ is also suggested in pituitary gonadotropes (Zhu et al., 2002), chromaffin cells (Yang et al., 2002), and in INS-1 cells (Yang and Gillis, 2004). The results reveal a novel mechanism to increase the apparent Ca^{2+} dependence

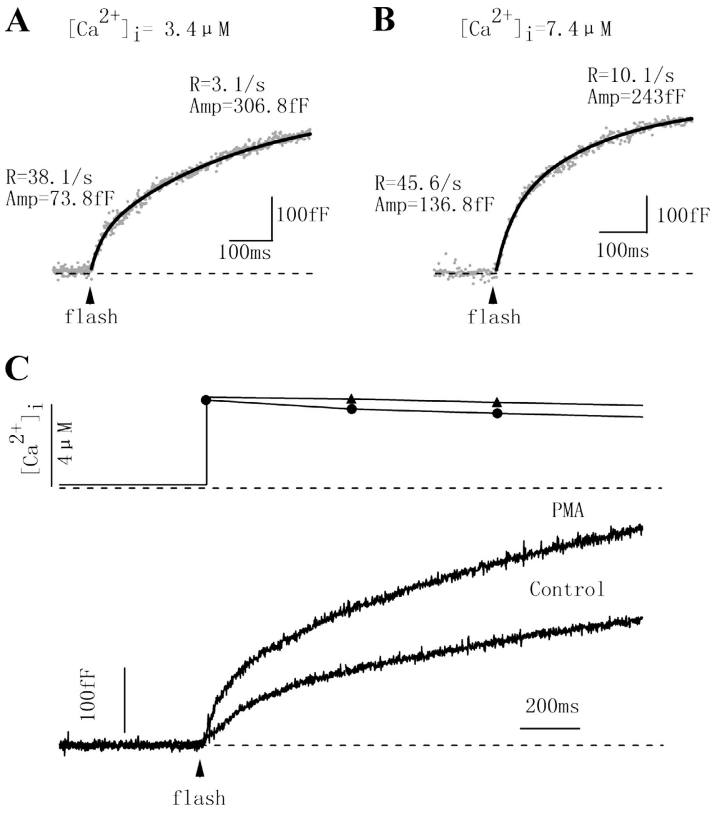

FIGURE 2. PMA increases the size of the HCSP. (A and B) Examples of the kinetics of exocytosis from PMA-treated (100 nM for 2–3 min) cells at two different post-flash $[Ca^{2+}]_i$ levels. Superimposed solid curves are exponential fits. (C) Averaged $[Ca^{2+}]_i$ (upper traces) and C_m responses (lower traces) from control (circles, $n = 14$) and PMA-treated (triangles, $n = 16$) cells.

of insulin release by recruiting more vesicles into a highly calcium-sensitive state.

Specificity of PMA Action in Regulating Insulin Secretion

PMA activates PKC due to its structural similarity to the endogenous activator, DAG, by binding to the C1 domain of PKC. The specificity of PMA activation of PKC has been questioned because PMA also activates protein kinase D, RasGRPs, and diacylglycerol kinase with equal potency (Kazanietz, 2002). Munc13, a family of proteins that prime exocytosis, also possess a PMA-binding (C1) domain and are translocated to the plasma membrane upon bath application of PMA (Rhee et al., 2002). Moreover, a recent report demonstrates that Munc13-1 functions in regulating insulin secretion (Sheu et al., 2003). Thus, we applied a number of specific PKC inhibitors to determine whether PKC is involved in the effect of PMA, and which PKC isoform might be involved in regulating secretion. Fig. 4 summarizes the averaged amplitudes of the HCSP and the RRP from control cells and cells treated with PMA and various PKC inhibitors. A specific pharmacological tool to test the involvement of PKC is the inhibitory peptide, PKC19-31, a pseudosubstrate sequence that interacts with the PKC substrate binding site in the C4 region of the catalytic domain. We included 1 μM PKC19-31 (IC_{50} = 100 nM) in the pipette solution and waited for 3 min after establishing the whole-cell configuration before flash. As shown in Fig. 4 A, PKC19-31 abolished the stimulatory effect of PMA on the HCSP.

At least five PKC isoenzymes (α, βΠ, δ, ε, and ζ) have been found in rat pancreatic β-cells (Kaneto et al., 2002). PMA activates the Ca^{2+}-dependent isoforms PKCα and PKCβII, and the Ca^{2+}-independent isoforms PKCδ and PKCε (Csukai and Mochly-Rosen, 1999). Here, we tried to better define the relevant PKC isoforms that are involved in regulating insulin secretion by comparing the differential effects of Gö6976 and Gö6983. Gö6976 selectively inhibits Ca^{2+}-dependent PKCα (IC_{50} = 2.3 nM) and PKCβI (IC_{50} = 6.2 nM), whereas it does not affect the kinase activity of the Ca^{2+}-independent PKC isozymes even in the micromolar range (Gschwendt et al., 1996). Gö6983 selectively inhibits several PKC isozymes but does not discriminate between them. As shown in Fig. 4 A, Gö6976 (100 nM) and Gö6983 (100 nM) were equally potent in blocking the stimulatory effect of PMA.

PMA enhanced the size of the RRP and the sustained component to a much lesser extent than its effect on the HCSP. This effect was also blocked by various PKC inhibitors (Fig. 4 A). Furthermore, we tested whether the PKC inhibitors used in this study could exert any effect on exocytosis in the absence of PMA. Fig. 4 B reveals that PKC19-31, Gö6976, and Gö6983 did not in-

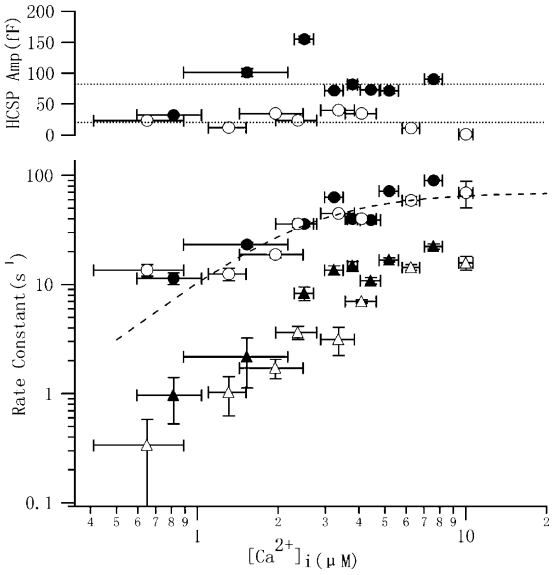

FIGURE 3. The rate constants and amplitudes of exponential fits to C_m responses with similar post-flash $[Ca^{2+}]_i$ levels are averaged and plotted versus $[Ca^{2+}]_i$. Circles and triangles represent the fast and slow components of double exponential fits. For $[Ca^{2+}]_i >$ 10 μM, it is hard to distinguish the HCSP from RRP, therefore we only fit a single exponential to the C_m traces and the rate constants are denoted as squares. The open and filled symbols represent data from control and PMA-treated cells, respectively. The Ca^{2+} dependence of the rate of exocytosis from the HCSP was fitted by the equation (dashed line) Rate = $R_{max}/(1 + (K_d/[Ca^{2+}]_i)^n)$, where R_{max}, K_d, and n were 69.4 ± 8.8 s^{-1}, 2.5 ± 0.5 μM, and 1.9 ± 0.7, respectively. The dotted lines in the upper panel mark the averaged HCSP sizes for control and PMA-treated cells.

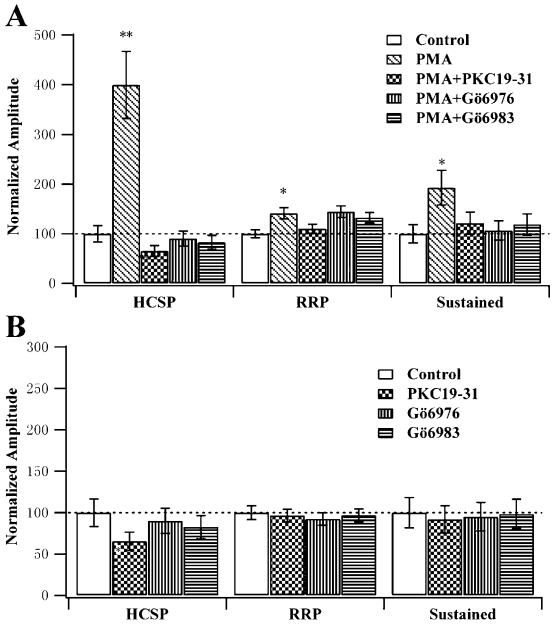

FIGURE 4. The effect of PMA is mediated by PKC activation. (A) Averaged amplitudes of different kinetic components for control cells and cells with various treatments. The C_m response was fitted by triple exponentials and the amplitudes of the three components were taken as the size of the HCSP, the RRP, and the sustained component. PKC19-31 (1 μM, $n = 19$), Gö6976 (100 nM, $n = 22$), or Gö6983 (100 nM, $n = 14$) were included in the pipette solution during perfusion with PMA in the external solution. (B) The effect of PKC19-31 ($n = 21$), Gö6976 ($n = 12$), or Gö6983 ($n = 19$) alone on the three kinetic components of exocytosis. Values represent the mean ± SEM. Asterisks denote significant differences (*t*-test, *P < 0.05, **P < 0.01). The sizes of the HCSP, RRP, and sustained components were normalized to their control values from paired experiments, respectively, to guard against day-to-day variation.

fluence the different components of secretion significantly, suggesting little tonic activity of PKC.

The Effect of PKA on Different Exocytotic Components in β-cells

To investigate the role of PKA in insulin secretion, we studied the effects of forskolin, an activator of adenylate cyclase, on the different secretory components in pancreatic β-cells. After application of 10 μM forskolin for 2–3 min, the amplitude of the HCSP was greatly enhanced (Fig. 5, A and B, see Fig. 1 for comparison). The averaged ΔC_m response to similar $[Ca^{2+}]_i$ levels showed a pronounced increase in amplitude over that from control cells (Fig. 5 A). As with PMA, forskolin did not change the kinetics of the secretory response, whereas it increased the size of the HCSP (Fig. 6 A). The Ca^{2+} dependence of the release rate from the HCSP and RRP was fitted to the same equation as in Fig. 3. As summarized in Fig. 6 (B and C), forskolin dramatically increased the size of HCSP as well as RRP. The stimulatory effect of forskolin was blocked by a PKA antagonist, Rp-cAMP (10 μM), demonstrating the involvement of PKA activation. Control experiments showed that Rp-cAMP had no effect on exocytosis in the absence of forskolin.

Convergent Effects of PKA and PKC on Exocytosis

To test whether PKC and PKA act on the same targets in enhancing exocytosis, we challenged pancreatic β-cells with combined application of 100 nM PMA and 10 μM forskolin in the bath solution and compared their effect with that of PMA or forskolin applied alone. As shown in Fig. 7, the cocktail of PMA plus forskolin exerted no greater effect than each compound alone. Thus, the effects of PKC and PKA are not additive, suggesting that activation of either one may converge on the same secretory pathway in the regulation of insulin secretion.

The Relationship between the HCSP and Rapid Depolarization-evoked Exocytosis

A subset of vesicles has been postulated to colocalize with Ca^{2+} channels and is often termed the immediately releasable pool (IRP) of vesicles (Horrigan and Bookman, 1994). The size of the IRP in pancreatic β-cells has been estimated to be around 30 fF (Barg et al.,

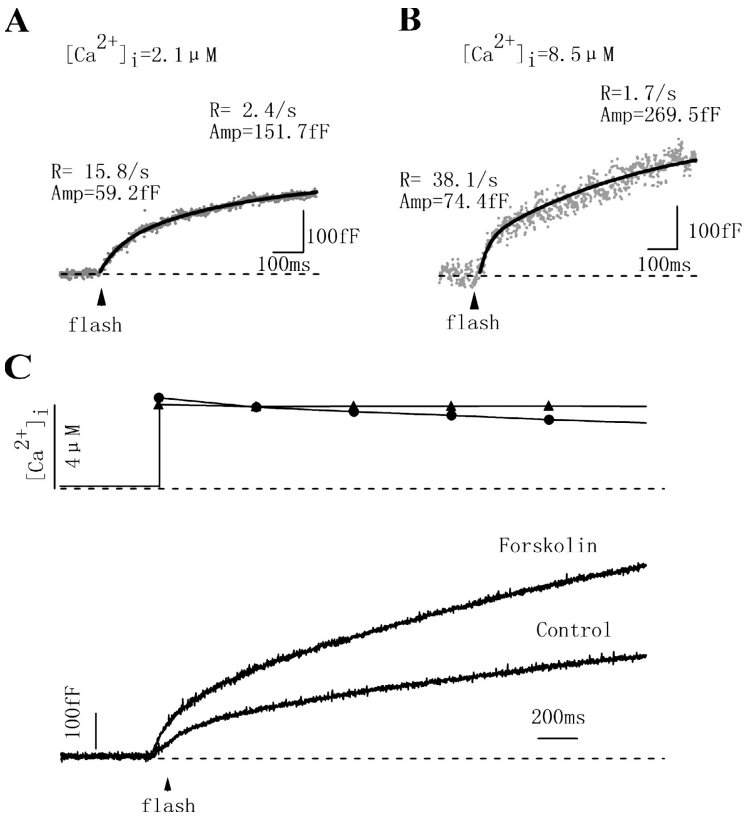

FIGURE 5. Forskolin significantly increases the size of the HCSP. (A and B) Examples of the kinetics of exocytosis from forskolin-treated cells (10 μM for 2–3 min) at two different post-flash $[Ca^{2+}]_i$ levels. Superimposed solid curves are exponential fits. (C) Averaged $[Ca^{2+}]_i$ (upper traces) and C_m responses (lower traces) from control and forskolin-treated cells, from experiments that had similar post-flash $[Ca^{2+}]_i$ values (n = 14 for control, circles; n = 13 for forskolin, triangles).

2001), which is similar to the basal size of the HCSP in β-cells. To clarify the relationship between the HCSP and the IRP, we have designed a cross-depletion experiment by combining brief membrane depolarization and flash photolysis of caged Ca^{2+}. We employed a dual-pulse protocol (two successive 30 ms depolarizing pulses separated by 100 ms at holding potential) to estimate the size of the IRP as previously described (Gillis et al., 1996), then a subsequent flash uncaging to elicit release from the HCSP. One example response in the presence of forskolin is depicted in Fig. 8 A, where elevation $[Ca^{2+}]_i$ to 4.3 μM is still capable of eliciting robust exocytosis from the HCSP after the depletion of IRP. Next, we repeated the cross-depletion protocol in the presence or absence of forskolin to determine whether the IRP and HCSP are differentially regulated. Fig. 8 B summarizes the results from 35 cells. The size of the IRP has not been significantly enhanced upon treatment of forskolin; however, the size of the HCSP following the depletion of the IRP was increased from 19.3 ± 4.5 to 78.4 ± 18.0 fF. This result further suggests that granules in the HCSP are distinct from those in the IRP.

DISCUSSION

We have characterized in detail the secretory response in single rat pancreatic β-cells to different $[Ca^{2+}]_i$ levels. We find a highly Ca^{2+}-sensitive phase of exocytosis in mouse β-cells in addition to the previously described RRP and reserve pool of vesicles. We have shown that application of either PMA or forskolin dramatically increases the size of the HCSP by a factor of approximately four, an effect involving activation of PKC or PKA as testified by the effectiveness of specific protein kinase inhibitors. The effects of PKC and PKA were not additive, suggesting that a convergent mechanism is used to modulate the secretory machinery in pancreatic β-cells.

$[Ca^{2+}]_i$ Dependence of Exocytosis in Pancreatic β-cells

The Ca^{2+} dependence of insulin secretion is controversial. It has been reported that little exocytosis is evoked in pancreatic β-cells upon photolysis of caged Ca^{2+} to levels $<\sim$3 μM (Takahashi et al., 1997; Barg et al., 2001), yet we detect robust exocytosis at $[Ca^{2+}]_i < 1$ μM. It is quite possible that the HCSP we report here has been overlooked in previous studies on β-cells because of its small and variable amplitude, yet we also see robust release from the larger RRP at 1 μM. Our results are consistent with a number of studies that have demonstrated insulin secretion at $[Ca^{2+}]_i$ levels of ~1 μM (e.g., Bergsten, 1995; Bokvist et al., 1995; Proks et al., 1996; Lang et al., 1997). One possible reason for the discrepancy between our results and other caged Ca^{2+} studies are differences in the $[Ca^{2+}]_i$ level before the flash and/or the presence of ATP in the pipette so-

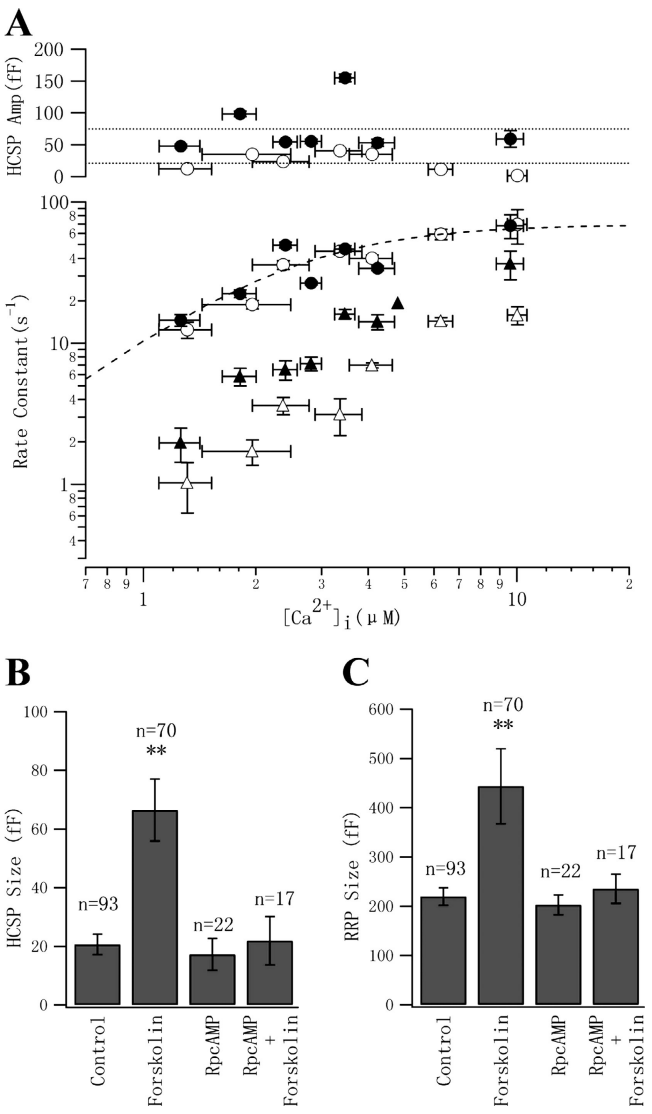

FIGURE 6. Summary of the effects of forskolin on different components of exocytosis. (A) The rate constants and amplitudes of exponential fits to C_m responses plotted versus $[Ca^{2+}]_i$. Circles and triangles represent the fast and slow components of double exponential fits. The open and filled symbols represent data from control and forskolin-treated (10 μM) cells, respectively. Squares denote the rate constants of single exponential fit to the C_m traces for $[Ca^{2+}]_i > 10$ μM. The dashed line is reproduced from Fig. 3. The lines in the upper panel mark the averaged HCSP size for control and forskolin-treated cells. (B) Comparison of the amplitude of the HCSP between control cells and those treated with forskolin, Rp-cAMP (10 μM) alone, or Rp-cAMP plus forskolin. (C) Application of forskolin significantly increased the amplitude of the RRP. Data are displayed as mean ± SEM. $^{**}P < 0.01$.

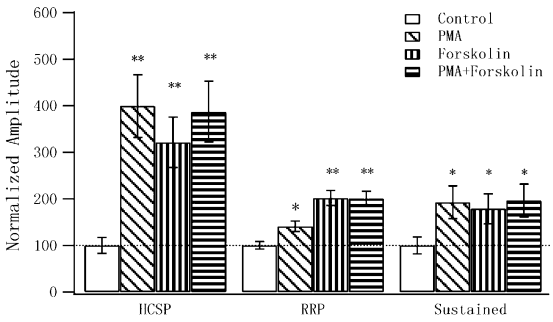

FIGURE 7. The effects of PMA and forskolin on exocytosis are not additive. Application of forskolin and PMA produced a fourfold increase in the size of the HCSP. PMA and forskolin also increased the size of RRP and the sustained component; however, the augmentation was not as dramatic as that of the HCSP. The sizes of the HCSP, RRP, and sustained components were normalized to their control values from paired experiments.

lution. For example, in the study by Takahashi et al. (1999), the pipette solution contained 10 mM DM-nitrophen together with 0.5–4 mM $CaCl_2$ and no ATP. The basal $[Ca^{2+}]_i$ was estimated to be <5 nM. In the present study we have determined the basal $[Ca^{2+}]_i$ to be ~200 nM and we included 2 mM ATP in the pipette solution. It should be noted that both basal Ca^{2+} and ATP are important for the priming and maintenance of the docked RRP (von Ruden and Neher, 1993; Parsons et al., 1995; Eliasson et al., 1997; Xu et al., 1998; Smith et al., 1998; Takahashi et al., 1999). Thus, our results supported the notion that pancreatic β-cells possess a high affinity Ca^{2+} sensor for exocytosis.

PKC Isoforms Involved in Insulin Regulation

PMA has been employed to investigate the role of PKC in the regulation of insulin secretion. However, because of the ubiquitous expression of many PKC isoforms and the large number of PKC regulators and substrates, the precise role of PKC activity and the identity of the relevant PKC isoforms have often remained elusive. Recently, Munc13s have been suggested as alternative DAG and Ca^{2+} receptors that function in regulating vesicle priming (Rhee et al., 2002). Thus, we used various specific PKC inhibitors including PKC19-31, Gö6976, and Gö6983 to demonstrate that PKC was indeed involved in the enhancement of exocytosis in β-cells. Among multiple PKC isoforms, only classical PKCs (α, βI, βΠ, and γ) and atypical PKCs (ζ, λ/ι, and μ) can be activated by PMA. BIS and the BIS-derived PKC inhibitor, Gö6983, inhibit several PKC isoforms (α, β, γ, δ, and ζ) without discriminating between them. Gö6976 selectively inhibits Ca^{2+}-dependent PKCα (IC_{50} = 2.3 nM) and PKCβI (IC_{50} = 6.2 nM), whereas it does not affect the kinase activity of the Ca^{2+}-independent PKC isoforms (δ, ε, and ζ) even in the micromolar range (Gschwendt et al., 1996). The fact that Gö6976 and Gö6983 were equally effective in blocking the stimulatory effect of PMA on the HCSP suggested the involvement of classical PKCs. Among all PKC isoforms, PKCα, βΠ, δ, ε, ζ, and ι are reported to be expressed in rat pancreatic islets. This narrows the PKC isoforms to PKCα and PKCβΠ. PKCα has been proposed in the priming of synaptic vesicles in the Calyx of Held (Wu

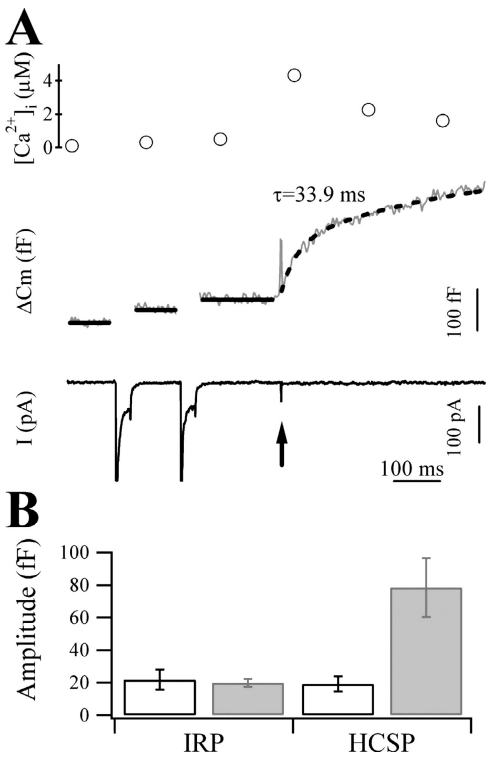

FIGURE 8. The HCSP is distinct from the IRP. (A) Sample response to two successive brief depolarizing pulses (30 ms in duration) to +10 mV followed by flash photolysis of caged Ca^{2+}. Note that the ΔC_m response to the second pulse is substantially smaller than the response to the first pulse, demonstrating the depletion of the IRP. At the time indicated by the arrow, a UV flash was given to elevate $[Ca^{2+}]_i$ to 4.3 μM. The dashed line is single-exponential fit with the fastest time constant indicated. The experiment is performed in the presence of 10 μM forskolin. (B) Summary of the estimated size of IRP and HCSP in the absence (open bars, $n = 8$) and presence of forskolin (filled bars, $n = 27$). The size of the HCSP is significantly increased by forskolin application (t test, $P < 0.05$), whereas the size of the IRP remains unchanged.

and Wu, 2001), whereas PKCβΠ is thought to induce c-myc expression and suppress insulin gene transcription (Kaneto et al., 2002). Recent experiments also have suggested that PMA-stimulated insulin secretion involved activation of PKCα but not PKCδ (Carpenter et al., 2004). Thus, we suspect that PKCα is involved in the enhancement of the HCSP in rat pancreatic β-cells.

Substrates of Protein Kinases

Identification of kinase substrates and of their cellular functions is crucial to a full understanding of the regulatory roles of protein kinases in insulin secretion. A number of unidentified kinase substrates have been localized to β-cell secretory vesicles or membrane fractions, which might be involved in vesicle trafficking, priming, and fusion. In this study, we have restricted the kinase substrates to those that are important for regulating vesicle priming and fusion by using patch-clamped β-cells and intracellular Ca^{2+} photorelease techniques. We found that activation of either PKA or PKC might act on a common site for changing the Ca^{2+} sensitivity of the primed vesicles. Despite a common effect on the HCSP, stimulation of PKA by 10 μM forskolin gives a greater enhancement of the RRP than stimulation of PKC by 100 nM PMA in our hand. Considering synaptotagmin and the soluble *N*-ethylmaleimide–sensitive fusion attachment protein receptor (SNARE) complex as an integrated calcium sensor for fusion (Xu et al., 1998), one could envision that phosphorylation of synaptotagmin or SNARE proteins, or other SNARE-interacting proteins, might finely adjust the Ca^{2+} sensing of exocytosis. The refilling and priming of vesicles involves multiple possible downstream effectors of protein kinases including SNARE proteins, αSNAP, and Rab proteins, etc. Overexpression of phosphomimetic and phosphorylation-defective mutant variants of SNAP-25 has recently dissected the role of PKC-mediated phosphorylation of SNAP-25 during vesicle recruitment in chromaffin cells (Nagy et al., 2002). Similar methods can be employed to investigate the substrates of protein kinases in β-cells. Alternatively, a more systematic proteomic approach will help to elucidate the kinase targets that are important in the regulation of insulin secretion.

Model of Insulin Secretion Control by Protein Kinases

Our study has revealed a novel, small HCSP with high Ca^{2+} sensitivity in rat pancreatic β-cells. The origin of this HCSP remains to be revealed since small GABA-containing synaptic-like vesicles have been suggested in pancreatic β-cells in addition to dense-core insulin-containing granules (Thomas-Reetz and De Camilli, 1994; Takahashi et al., 1997). However, recently the release of GABA-containing synaptic-like vesicles has been estimated to contribute only ∼1% of the capacitance signal in β-cells (Braun et al., 2004). In an accompanying report, Yang and Gillis (2004) have shown that quantal 5-HT release correlates well with the exocytosis HCSP in insulin-secreting INS-1 cell line. Simultaneous measurement of insulin and 5-HT release with modified carbon fiber electrodes demonstrates that 5-HT is released exclusively from insulin-containing granules (Aspinwall et al., 1999). Thus, it is possible that the HCSP is composed of the same type of insulin-containing granules as the "conventional" RRP.

We have shown that PKC and PKA activation can dramatically increase the size of the HCSP. Similar effects have also been reported in the rat insulinoma INS-1 cells (Yang and Gillis, 2004), albeit augmentation of the fraction of granules in the HCSP was less prominent in this study. The preferential augmentation of the HCSP has been also reported in chromaffin cells (Yang et al., 2002) and possibly in INS-1 cells when

stimulated with glucose (Yang and Gillis, 2004). We have found that the bulk of the HCSP is not released in response to brief depolarization sufficient to deplete the IRP, suggesting that most of the granules in the HCSP do not colocalize with Ca^{2+} channels. The same conclusion has been drawn from studies in pituitary gonadotropes (Zhu et al., 2002), chromaffin cells (Yang et al., 2002), and INS-1 cells (Yang and Gillis, 2004). Thus, the HCSP is likely to respond to global elevation of $[Ca^{2+}]_i$ rather than localized Ca^{2+} microdomains. A preferential enhancement of the HCSP would mean that insulin secretion can be potentiated at substimulatory $[Ca^{2+}]_i$ values upon activation of intracellular protein kinases (Jones et al., 1985, 1986) This mechanism may also explain how PMA can give rise to a slowly developing component of insulin secretion even at a subthreshold glucose concentration (Bozem et al., 1987). It is plausible that both nutrient and nonnutrient secretagogues might modulate insulin secretion by recruiting more granules into the HCSP through activation of PKA and PKC.

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