Potentiation of insulin secretion by phorbol esters is mediated by PKC-α and nPKC isoforms

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Yaney, Gordon C., Jamison M. Fairbanks, Jude T. Deeney, Helen M. Korchak, Keith Tornheim, and Barbara E. Corkey. Potentiation of insulin secretion by phorbol esters is mediated by PKC-α and nPKC isoforms. Am J Physiol Endocrinol Metab 283: E880–E888, 2002. First published July 30, 2002; 10.1152/ajpendo.00474.2001.—Culturing clonal β-cells (HIT-T15) overnight in the presence of phorbol ester [phorbol myristate acetate (PMA)] enhanced insulin secretion while causing downregulation of some protein kinase C (PKC) isoforms and most PKC activity. We show here that this enhanced secretion required the retention of PMA in the cell. Hence, it could not be because of long-lived phosphorylation of cellular substrates by the isoforms that were downregulated, namely PKC-α, -βII, and -ε, but could be because of the continued activation of the two remaining diacylglycerol-sensitive isoforms δ and μ. The enhanced secretion did not involve changes in glucose metabolism, cell membrane potential, or intracellular Ca2+ handling, suggesting a distal effect. PMA washout caused the loss of the enhanced response, but secretion was then stimulated by acute readdition of PMA or bombesin. The magnitude of this restimulation appeared dependent on the mass of PKC-α, which was rapidly resynthesized during PMA wash-out. Therefore, stimulation of insulin secretion by PMA, and presumably by endogenous diacylglycerol, involves the activation of PKC isoforms δ and/or μ, and also PKC-α.

in either the action of glucose to stimulate insulin secretion or in potentiating such secretion (21, 27, 28). Two potentiating agents, acetylcholine or cholecystokinin, are widely held to augment the effect of nutrient secretagogues by triggering the generation of DAG and the activation of PKC (10, 32), indicating that the activation of PKC provides a positive signal for insulin secretion.

The complexity of signal transduction via PKC is increased considerably by the existence of 11 isoforms. These isoforms differ in their requirements for the activators, Ca2+ and lipids, and in their intracellular location (19). The known PKC isoforms can be divided into the following three major classes: the conventional (cPKC), which are activated by both Ca2+ and DAG; the novel (nPKC), which require only DAG; and the atypical (aPKC), which bind neither Ca2+ nor DAG. Clonal β-cells (HIT-T15 and MIN6) have been shown to express seven PKC isoforms, including cPKC (α and βII), nPKC (δ, ε, and μ), and aPKC (λ and ζ; see Refs. 26 and 29). Phorbol esters, such as phorbol myristate acetate (PMA), can substitute for DAG as high-affinity ligands for cPKC and nPKC isoforms, and the acute addition of PMA to β-cells stimulates secretion (7). Although most isoforms in other kinase families share a similar activation mechanism, this is clearly not the case for PKC. This observation has led to the assumption that the different PKC isoforms underpin different functions within a given cell (15). However, very little is known about the role of PKC isoforms that are expressed in β-cells and specifically how and which PKC isoforms couple intracellular lipid signals to the stimulation of exocytosis (26, 29).

Downregulation of PKC activity, because of chronic stimulation (12–24 h) by phorbol esters, has been used widely as a tool to examine the involvement of PKC in insulin secretion (1, 2, 13, 17, 18, 25, 26, 29, 31). Some studies have treated PKC as a single enzyme activity and have correlated the reduction of total PKC activity to a given change in the secretory response of the β-cell (1, 2, 13, 17, 18, 25). More recent studies have demon-
stated that chronic stimulation by phorbol ester resulted in the differential downregulation of PKC isoform mass (26, 29). In HIT cells, overnight exposure to PMA selectively downregulated PKC-α, -βII, and -ε while preserving PKC-δ, -μ, -γ, and -ζ. This differential loss of PKC isoform mass was correlated with the loss of Ca^{2+}-sensitive PKC activity (29). Interestingly, not only did chronic PMA treatment and PKC downregulation not diminish glucose-stimulated secretion but they resulted in an enhanced secretory response to stimulation by glucose, cell depolarization, or cholinergic stimulation, suggesting an increased sensitivity in a signaling step common to various secretagogues (17, 29, 31). The mechanism of this increased secretion is unknown but has been previously attributed to either the removal of an inhibitory input by PKC or the long-lived phosphorylation of cellular substrates by the activated and subsequently downregulated isoforms, in particular PKC-α (17, 31).

The studies presented here support a different explanation. After chronic PMA treatment, β-cells (HIT-T15) no longer responded to the acute addition of PMA but exhibited a heightened release of insulin, which required the presence of residual PMA in the cell. These data indicated that increased secretion was the result of the continued PMA-induced activity of one or more of the DAG-sensitive PKC isoforms that are not downregulated. Removal of the residual cellular PMA after downregulation caused a slight resynthesis of PKC-α and -ε. The functional consequence of PKC resynthesis was that acute PMA again stimulated secretion, and the magnitude of this response correlated with changes in the mass of PKC-α, but not PKC-ε. A similar correlation was observed for the mass of PKC-δ and/or -μ. These extracts included [3H]PMA contained within the cells and bound to the plate itself. In these initial experiments, no correction was made for [3H]PMA binding to plastic.

After this initial experiment, the removal of PMA was optimized by using 6- or 12-well plates. Once the optimal concentration of BSA was determined, HIT cells were grown in 12-well plates and downregulated using standard RPMI 1640 supplemented with 10% FCS and 200 nM PMA for ≥18 h. Removal of PMA was accomplished over the course of 1 h by repeated washes at 5-min intervals with preheated KRB (37°C) containing 1% fatty acid-free BSA. By use of these conditions, there was no difference between 1 and 3% BSA in the rate of PMA removal. Correction for [3H]PMA bound to the plastic itself was determined using the same set-up in plates without cells. At 60 min, ~90% of the bound PMA was removed, with one-half of the remaining radioactivity being accounted for by PMA binding to the empty plastic well. After the washout of PMA, the cells were assayed for insulin secretion using a 30-min incubation as outlined previously.

Preparation of cell samples and SDS-PAGE. Cells were extracted in ice-cold 50 mM Tris-HCl, pH 7.5, containing 5 mM EGTA, 1 mM phenylmethylsulfonlfyl fluoride, 50 mM 2-mercaptoethanol, and 25 μg/ml each of leupeptin and aprotinin. Cells were collected from three replicate wells with 300 μl extraction buffer by scraping and sonicated on ice. Sonication was done with a Branson Cell Disruptor using a micro-tip at a power level of two using two 6-s pulses at 50% duty cycle. The mixture was spun for 10 min at 700 g to remove cellular debris and nuclei. The protein content of these homogenates was determined using Bio-Rad reagent. SDS-PAGE was performed according to the method of Laemmli (16).

Western blotting. Transfer of protein to nitrocellulose paper was done electrophoretically using a semidry apparatus from Owl Scientific (Cambridge, MA). A transfer buffer of Tris-HCl, pH 7.4, containing SDS and methanol was used, and the transfer was performed with constant current at room temperature. Blots were probed with isozyme-specific polyclonal antibodies as outlined by the various suppliers (GIBCO Life Sciences, Santa Cruz Biochemicals, and Transduction Laboratories). The secondary antibody was a goat anti-rabbit IgG conjugated to horseradish peroxidase purchased from Boehringer Mannheim. The specificity of the interaction was assessed by using the isozyme-specific block- ing peptide provided. Visualization of the binding of the horseradish peroxidase-conjugated secondary antibody was achieved using the enhanced chemiluminescence (ECL) kit from Amersham.

Measurement of intracellular free Ca^{2+}. Cytosolic free Ca^{2+} was measured in suspensions of cells loaded with the Ca^{2+} indicator fura 2 by use of the ratios method, as described previously (6).
Measurement of extracellular free Ca\(^{2+}\). Free Ca\(^{2+}\) in RPMI 1640 used during PMA-induced downregulation of PKC isoforms was measured in the presence or absence of 425 \(\mu\)M EGTA. Measurements were made using an Orion Ca\(^{2+}\) selectrode (Boston, MA) attached to an amplifier from the Biomedical Instrumentation Group (University of Pennsylvania School of Medicine) and standards provided by World Precision Instruments (Sarasota, FL).

Monitoring changes in membrane potential. Changes in cellular membrane potential were monitored using the cell-permeant indicator bis-oxyxonol (excitation, 540 nm; emission, 590 nm), as previously outlined (30).

Monitoring changes in cellular NADH. The changes in cellular NADH levels were monitored by measuring the change in its intrinsic fluorescence using excitation and emission wavelengths of 340 and 460 nm, respectively (4). These changes were expressed as a percentage of the full scale determined from a minimum seen with the uncoupling agent FCCP (50 nM) and the maximum obtained using the respiratory blockers antimycin A (0.5 \(\mu\)M) and oligomycin (2.5 \(\mu\)g/ml).

Scanning and densitometry of SDS-PAGE. Films exposed to ECL were scanned, and the image was captured using an Agfa StudioStar flatbed scanner in conjunction with PhotoLook for Windows (version 2.09). The digitized image was analyzed using Scion Image for Windows (version 4.1), which is derived from NIH Image for Macintosh. Comparison of band density was done only within a single film, reflecting samples run on the same gel.

Statistics. The data are reported as means and SE of individual observations. Significance was assessed by Student’s t-test and considered significant at \(P<0.05\). Multiple comparisons were examined using a one-way ANOVA with a Student-Newman-Keuls test done post hoc. Differences were considered significant at \(P<0.05\).

RESULTS

Enhanced secretion seen after overnight PMA treatment and PKC downregulation. Basal, glucose-stimulated, and KCl-induced insulin secretion was not only preserved but was enhanced in clonal \(\beta\)-cells (HIT-T15) after overnight PMA-induced downregulation of PKC (Fig. 1). Like glucose, 30 mM KCl-induced depolarization of the \(\beta\)-cell stimulates secretion by activating L-type and other voltage-dependent Ca\(^{2+}\) channels and raises \([\text{Ca}^{2+}]_i\) (30). The enhanced secretory response to KCl in clonal \(\beta\)-cells seen here (2-fold) was similar to the 2.5-fold stimulation previously described in islets cultured overnight in the presence of phorbol ester (31). However, in this clonal cell line, unlike islets, KCl elicited a greater secretory response than glucose. The inactive phorbol ester phorbol 12,13-didecanoate (4\(\alpha\)-PDD) was compared with its active stereoisomer 4\(\beta\)-PDD and PMA. Only prolonged exposure to 4\(\beta\)-PDD and PMA resulted in the enhanced response seen with downregulation and was characterized by the lack of response to the acute addition of PMA (data not shown).

The time course of PKC isoform downregulation is shown in Fig. 2. The \(\beta\)II isoform was most rapidly downregulated, becoming undetectable by Western blotting within 3 h of PMA exposure. PKC-\(\alpha\) and -\(\epsilon\) became undetectable some time between 6 and 9 h after PMA addition. These three isoforms remained undetectable after overnight exposure to PMA, indicating that they were not involved in the enhanced secretion observed after overnight PMA treatment (Fig. 1). Consistent with previous reports, the remaining four PKC isoforms (PKC-\(\delta\), -\(\mu\), -\(\tau\), and -\(\zeta\)) were not altered during this time course or after overnight exposure to PMA (data not shown; see Refs. 26 and 29).

Effect of preserving insulin content. Because of the stimulatory effect of PMA on insulin secretion, insulin content decreases during overnight PMA treatment. To minimize the loss of insulin content during downregulation and to ensure that control and downregulated cells were well matched, extracellular \([\text{Ca}^{2+}]_i\) was reduced in the culture medium to blunt insulin secretion. Results in Table 1 demonstrate that the addition of EGTA to the medium reduced Ca\(^{2+}\) levels from 875 to 155 \(\mu\)M and preserved \(\beta\)-cell insulin content during chronic PMA treatment and PKC downregulation. In contrast, the insulin content of \(\beta\)-cells chronically treated in the presence of normal levels of extracellular Ca\(^{2+}\) was reduced by 29% compared with controls. Comparison of secretion from \(\beta\)-cells having normal and reduced insulin content demonstrated that preservation of insulin content increased the secretory responses (Fig. 3). KCl-induced secretion was used for this comparison because of its large stimulation of secretion before and after PKC downregulation (Fig. 1). The increased response obtained by preserving insulin content was seen under basal conditions, with KCl
alone, or in combination with glucose and forskolin, an agent that greatly increases cAMP. These results demonstrate that loss of stored insulin decreases the responses to secretagogues and thereby emphasize the importance of matching insulin content so as not to underestimate the effect of chronic PMA treatment on secretion.

**Enhanced secretion after downregulation requires PMA retention.** Because phorbol esters are lipophilic and DAG-sensitive PKC isoforms remain after downregulation, a possible reason for the enhanced secretion observed might be the retention of cellular PMA. Therefore, we compared the secretion from downregulated β-cells with preserved insulin content (Fig. 3, reduced Ca\(^{2+}\)) with the response of control β-cells in which 200 nM PMA was added acutely (Fig. 3, acute PMA). Although the degree of increase in secretion resulting from KCl-induced depolarization was large, it was not maximal, as judged by the further response to the combination of glucose, KCl, and forskolin. Furthermore, the response to KCl or the combination of secretagogues was similar in these two groups, suggesting that the enhanced response to KCl in the overnight-treated cells, shown in Fig. 1, could be because of retention of cellular PMA. In contrast, basal insulin secretion in acutely stimulated cells was greater than that seen in downregulated cells with or without normal insulin content (Fig. 3).

![Graph](https://example.com/graph.png)

**Table 1. Effect of decreased extracellular Ca\(^{2+}\) on insulin content during chronic PMA treatment and PKC downregulation**

| Downregulation Conditions | Medium \([\text{Ca}^{2+}]\), \(\mu\text{M}\) | % Control (insulin content) |
|---------------------------|--------------------------------|
| No PMA/EGTA               | 155 ± 8 (5)                    | 100 (5)                     |
| PMA/EGTA                  | Not measured                   | 100 ± 8 (5)                 |
| PMA/no EGTA               | 875 ± 48 (5)                   | 71 ± 2 (2)                  |

Data are means ± SE; no. of experiments in parentheses. [\(\text{Ca}^{2+}\)], Ca\(^{2+}\) concentration. Insulin content was determined at the end of 24 h of phorbol myristate acetate (PMA) treatment. Free Ca\(^{2+}\) in RPMI 1640 used during PMA-induced downregulation of protein kinase C (PKC) isoforms was measured in the presence or absence of 425 \(\mu\text{M}\) EGTA using an Orion Ca\(^{2+}\) electrode. Control values for insulin content ranged from 83 to 221 ng/well. Comparisons reported in Figs. 5–8 used conditions of lines 1 and 2.

**Fig. 2. Time course of downregulation of PKC isoforms.** HIT cells were exposed to 200 nM PMA for various times before washing and homogenizing, as outlined in METHODS. After this, 20 \(\mu\text{g}\) protein were run on a gel and blotted as outlined in METHODS. Relative scanning density for each isoform along the time course measured is shown. Data are means ± SE; \(n = 3\). *\(P < 0.01\), mass of PKC-α vs. time 0. **\(P < 0.001\), mass of PKC-α vs. its mass at 3 h. +\(P < 0.01\), mass of PKC-ε vs. its mass at time 0. ++\(P < 0.05\), mass of PKC-ε vs. its mass at 3 h.

**Fig. 3. Protection of insulin content and secretory response.** Cells were downregulated with 200 nM PMA in RPMI 1640 with normal Ca\(^{2+}\) or RPMI 1640 containing 425 \(\mu\text{M}\) EGTA and normal Ca\(^{2+}\). Cells were given a 30-min preincubation under basal conditions followed by a 30-min stimulation by agents shown. Control cells were exposed to 200 nM PMA only acutely during stimulation. Basal, 0 glucose; high glucose (HG), 5 mM; KCl, 30 mM; forskolin, 10 \(\mu\text{M}\). Data are means ± SE; \(n = 6\). *\(P < 0.05\) compared with basal secretion/normal Ca\(^{2+}\). #\(P < 0.01\) compared with basal secretion/normal Ca\(^{2+}\). \#\(P < 0.01\) compared with HG+KCl+forskolin stimulation/normal Ca\(^{2+}\) or compared with HG + KCl + forskolin stimulation/normal Ca\(^{2+}\).
The rate of PMA removal was compared among various concentrations of fatty acid-free BSA in the media. The efflux rate of [3H]PMA was increased dramatically by increasing the BSA concentration between 0.05 and 1.5% (Fig. 4, inset). On the basis of these results, PMA washout was found to be optimal, with changes of KRB containing 1% fatty acid-free BSA every 5 min during the course of an hour-long washout (Fig. 4). Using this approach, ~90% of the PMA retained in the β-cells after downregulation was removed, and, of the remaining 10%, one-half of this was accounted for by PMA binding to plastic itself, which was removed during homogenization.

The enhanced secretion after downregulation was largely reversed within the hour required to remove PMA. Thus basal secretion was enhanced 2.6-fold (260 ± 22%, n = 7, P < 0.01) and upon PMA washout secretion returned to 1.3-fold of control (129 ± 12%, n = 7, P < 0.05). Similarly, glucose-stimulated insulin secretion was enhanced 2.2-fold (219 ± 22%, n = 7, P < 0.01) after downregulation and upon PMA washout returned to 1.2-fold of control (121 ± 12%, n = 7, P > 0.1). These data suggest that the enhanced secretion after PKC downregulation was mediated by a relatively short-lived phosphorylation because of the continued stimulation of the PMA-sensitive PKC isoforms δ and/or μ. The data also imply that addition of PMA after successful washout would restore enhanced secretion.

Readdition of PMA after its washout stimulated insulin secretion. A functional consequence of PMA-induced downregulation, without adequate washout, was that subsequent addition of phorbol ester did not further stimulate secretion (Fig. 5A, PMA retained). The comparison of control cells and downregulated cells with PMA retained demonstrated the expected potentiation of both basal secretion and glucose-stimulated insulin secretion without further stimulation of secretion by acute PMA. In contrast, when the PMA retained from overnight incubation was removed rapidly, basal and glucose-stimulated secretion returned to control levels, and the potentiation of glucose-stimulated insulin secretion by PMA was possible again. Readdition of an inactive phorbol ester had no effect on glucose-stimulated secretion (data not shown). Unexpectedly, with PMA washout, this repotentiation of glucose-stimulated secretion was greater than the enhanced secretion observed after PKC downregulation and matched the potentiation by PMA seen in control cells (Fig. 5A).

Readdition of PKC-α and PKC-ε. Western blots, paired to the three secretion conditions shown in Fig. 5A, indicated that PKC-α, PKC-βII, and PKC-ε were lost with downregulation and did not reappear with PMA removal (Fig. 5B). As expected, PKC-δ and -μ were not downregulated. However, if the blots of PKC-α, PKC-βII, and PKC-ε were overexposed, a
PKC-II was undetectable after PMA was evident, as seen in the bottom blot of each pair in Fig. 5. In all experiments, the response to glucose and PMA in condition 2 was closely matched to that of the control cells (condition 1). Inset: paired Western blots of PKC isoforms for conditions 1–3.

small, but detectable, resynthesis of PKC-α and PKC-ε was evident, as seen in the bottom blot of each pair in Fig. 5B. In contrast, PKC-βII was undetectable after downregulation and did not reappear during PMA washout. Therefore, we asked if blocking the resynthesis of α and ε would affect the potentiation of insulin secretion by the acute PMA.

To assess the effect of blocking the resynthesis of PKC-α and -ε, retained PMA was removed using buffers with or without 3 μg/ml cycloheximide, and secretion was compared between these two groups. One such comparison from a series of six separate experiments is shown (Fig. 6). In a recent study, cycloheximide used at 10 μg/ml had no effect on glucose-stimulated secretion after 1 h (11). In the experiment shown in Fig. 6, cycloheximide largely prevented the recovery of PMA potentiation of secretion. Western blots of PKC-α and -ε from cells used in the experiment are shown in Fig. 6, inset. Resynthesis of PKC-α and -ε was not detectable where PMA was removed in the presence of cycloheximide (washout condition 3), whereas both isoforms demonstrated some resynthesis when PMA was removed in its absence (condition 2). For comparison, the mass of these isoforms in control cells is shown in Fig. 6, inset, lane 1. It is noteworthy that in all experiments the mass of PKC-α or -ε after PMA downregulation was a very small fraction of its mass in control cells. PKC-δ and PKC-μ were unaffected by downregulation (data not shown). These data suggest that either PKC-α, PKC-ε, or both isoforms could be partially responsible for mediating the potentiation of glucose-stimulated secretion by the readdition of PMA.

In the experiments discussed above, the effectiveness of cycloheximide in preventing PKC-α and -ε resynthesis was variable. We took advantage of this variation to compare the effectiveness of PMA to replete glucose-stimulated insulin secretion (GSIS) with changes in the mass of these isoforms. In this comparison (Fig. 7), the correlation was considerably stronger for PKC-α \((r^2 = 0.88)\) than for PKC-ε \((r^2 = 0.24)\). In addition, the correlation for change in the combined mass of PKC-α and -ε and secretion was intermediate between either isoform alone \((r^2 = 0.65)\). These data indicated that the restimulation of insulin release by PMA correlates with resynthesis of the mass of PKC-α and not PKC-ε, although the mass of both isoforms varied over a similar range in these experiments.

To assess the effect of PKC-α resynthesis on the potentiation of GSIS resulting from the in situ generation of DAG, the action of the neuropeptide bombesin was examined in experiments analogous to those depicted in Fig. 6. As shown in Fig. 8, the potentiating action of bombesin was blunted significantly when, after downregulation, PMA was washed out in the presence (condition 3) vs. the absence of cycloheximide (condition 2). Western blotting (Fig. 8, inset) was used...
to compare the mass of PKC-α from cells not downregulated (condition 1) with those cells after PMA downregulation and washout (conditions 2 and 3). In the absence of downregulation, the ability of bombesin to increase intracellular Ca\(^{2+}\) and potentiate GSIS was also examined after exposure to cycloheximide. Bombesin (100 nM; Sigma) was added at the time of incubation. Insulin values compare secretion in downregulated cells in which PMA was washed out ± cycloheximide (n = 4). Experiments were repeated in another passage with similar results. *P < 0.01 compared with condition 2. Inset: Western blots of PKC-α for conditions 1, 2, and 3.

Second, the ability of increased extracellular KCl to depolarize the β-cell was measured using the potential-sensitive fluorescent probe bis-oxonol. When the increase in this signal was expressed as a percentage of the starting fluorescence value, the response of chronic PMA-treated cells was depressed significantly to 81 ± 7% of the control response (P = 0.02, n = 6; data not shown). However, this result is opposite to the change required for enhanced secretion.

Third, because both glucose and elevated extracellular K\(^{+}\) increase intracellular Ca\(^{2+}\), the effect of downregulation on [Ca\(^{2+}\)] was assessed. As expected, increases in [Ca\(^{2+}\)] and insulin secretion were seen with increasing extracellular KCl or glucose concentrations. However, although both KCl-induced and glucose-stimulated secretion was enhanced after PKC downregulation, increases in [Ca\(^{2+}\)] were the same (data not shown).

Last, the possibility that downregulation altered glucose sensitivity was evaluated. There was no difference in the concentration of glucose required for half-maximal secretion, with values being 0.7 ± 0.2 and 0.6 ± 0.2 mM for control and downregulated cells, respectively. It should be noted that the clonal β-cells used here (HIT-T15) are significantly left shifted in their response to glucose compared with primary cells.

Given these data, the probable site or sites of enhanced secretion seen after PMA-specific PKC downregulation would appear to be distal to cell metabolism, membrane depolarization, and the rise in intracellular Ca\(^{2+}\) caused by glucose or KCl stimulation.

**DISCUSSION**

Downregulation of PKC activity by chronic stimulation has been used in various cell types and by many investigators to provide evidence for the involvement of PKC in cell processes. Downregulation of PKC isoforms is thought to occur via activation and subsequent proteolysis at membrane surfaces and may depend on transactivation by other enzymes (12, 20). The conventional and novel isoforms, because of their sensitivity to phorbol esters, are activated and targeted to membranes where they may be degraded by Ca\(^{2+}\)-dependent proteases with prolonged PMA exposure.

Our recent work (29), and data presented here, have shown that chronic PMA treatment of clonal β-cells (HIT) resulted in the downregulation of three of the seven isoforms expressed. The PKC isoforms α, βII, and ε were undetectable after overnight exposure to PMA and correlated with the loss of PKC activity that was Ca\(^{2+}\)/DAG dependent (29). The nPKC isoforms δ and μ were resistant to downregulation. The resistance of PKC-δ to clearance could be specific to the β-cell (clonal or native), for it is cleared from other cell types after chronic PMA treatment (12). The presence of PKC-μ was previously reported in another β-cell line, MIN6, where it was also unaffected by chronic PMA treatment (26).

The functional result of this chronic PMA treatment was the enhancement of both KCl- and glucose-stimu-
lated insulin secretion in clonal β-cells (HIT-T15) and rat islets (29, 31). This was originally suggested to result from a long-lived phosphorylation of cellular substrates still remaining after downregulation of PKC-α (6, 31). However, the short time to downregulation and the rapid reversal of this enhancement on PMA removal, within 1 h, after the overnight PMA treatment are inconsistent with this proposal. Instead, these data indicate that the enhancement is because of the continued activation of one or both of the isoforms that were not downregulated but are PMA sensitive, namely δ and μ. Interestingly, MIN6 cells, which apparently do not contain PKC-δ, were reported not to exhibit enhanced secretion after downregulation (26), suggesting that the PKC-δ isoform is involved in the enhanced secretion exhibited by HIT cells and primary β-cells (31). Alternatively, a constitutively active PKC M-fragment produced by downregulation might underpin enhanced secretion, but this proposal is incompatible with the requirement for retained PMA, since this species is not activated by phorbol esters. In addition, PKC-μ and -ζ also remain after downregulation but cannot be activated by PMA. Thus the involvement of nPKC would be consistent with earlier studies where downregulation was assessed by the readdition of phorbol ester or the loss of cPKC isoforms. A non-PKC target of PMA is also a possibility. One such alternative is the C1 motif-containing presynaptic protein Munc13-1, which is expressed in neuronal cells and might be consistent with our data. This protein has been suggested to mediate the enhancement of neurotransmitter release by phorbol esters and DAG (3, 23).

The results presented here indicate that the enhanced secretion seen with glucose or KCl after chronic PMA treatment does not involve major changes in cell depolarization, Ca²⁺ handling, or glucose metabolism. Subtle changes in Ca²⁺ metabolism seen at the single-cell level may have been missed in these studies (1) but are unlikely to account for the enhanced responses observed. Because glucose-stimulated and KCl-induced secretion were similarly affected, it is likely that the enhanced secretion resulted from an alteration in the cellular response to a rise in intracellular Ca²⁺. Therefore, because this enhancement of secretion is likely the result of activation of nPKC isoforms, the results imply that at least these PKC isoforms affect a step in the stimulus-secretion coupling pathway down-stream from the Ca²⁺ rise.

The dependence of the enhanced secretion on the continued presence of PMA also argues against another previously proposed explanation, that the enhancement is the result of removal of an inhibitory action from one of the downregulated PKC isoforms (17). Furthermore, the enhanced secretion after downregulation was at best equal to the secretion of control cells acutely stimulated by PMA, when loss of insulin content was prevented (Fig. 3), indicating that removal of a negative input cannot be the primary explanation. Examination of the individual downregulated PKC isoforms did not suggest an inhibitory action. Thus the positive correlation of PKC-α mass with secretion indicated that this isoform has a positive input. There was no obvious correlation, either positive or negative, of secretion with resynthesized PKC-ε mass. Whether the third downregulated isoform, PKC-βII, has either positive or negative actions could not be ascertained, since it was not resynthesized.

The positive correlation of the potentiation of glucose-stimulated secretion with resynthesized PKC-α mass indicated that this isoform provided a second mechanism for coupling PMA/DAG to insulin release. This is consistent with PMA enhancing basal secretion more strongly in control cells than downregulated cells where PKC-α has been lost (Fig. 3). Our finding that the mass of PKC-α was limiting for restimulation of secretion by PMA (Fig. 7) is consistent with earlier studies showing that GSIS correlated with the translocation of PKC-α to the plasma membrane (8, 9). Perhaps it should be expected that PMA- and glucose-stimulated secretion would have a similar correlation with the mass of PKC-α based on the assumption that PMA and DAG activate the same subset of PKC isoforms. The present study, however, goes beyond those initial studies in that all of the isoforms expressed in the β-cells were examined, not just PKC-α. The correlation seen here with PKC-α was not seen with PKC-ε and could not be tested for PKC-βII, since the latter was not resynthesized after downregulation within the time frame examined.

Perhaps one of the most striking findings of this series of experiments was that resynthesis of even a small fraction of the PKC-α normally found in these cells appeared responsible for the recovery of normal stimulation of secretion by PMA (Fig. 5). Support for the specificity of this effect is provided by the observation that the resynthesis of PKC-ε under identical conditions did not correlate with potentiation of secretion by PMA. The fact that PKC-α mass must be reduced by >95% to be limiting suggests that only a small fraction of this isoform normally present is catalytically competent or involved in secretion. If so, this fact raises the possibility that the fraction of PKC-α involved in secretion is discreetly localized in the cell to support this function. The physiological relevance of this finding is seen in data demonstrating an analogous situation for the potentiation of GSIS by bombesin where retarding the resynthesis of PKC-α with cycloheximide also prevented restoration of bombesin sensitivity (Fig. 8). Previously, bombesin has been shown to cause a rapid (20-s), significant, and biphasic rise in both total DAG and inositol 1,4,5-trisphosphate in HIT cells (24). Potentiation of GSIS by bombesin is more complex than the addition of PMA, since phorbol esters do not increase intracellular Ca²⁺ in HIT cells. However, we have shown here that the ability of bombesin to increase intracellular Ca²⁺ and the ability of these cells to respond to this signal were not impaired by exposure to cycloheximide.

In conclusion, these data with clonal β-cells (HIT-T15) are consistent with a model of stimulated secretion where phorbol esters or endogenous DAG provide a positive input for secretion by the activation of both
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