Perspectives in Diabetes

Is Protein Kinase C Required for Physiologic Insulin Release?

STEWART A. METZ

Extant data suggest that a Ca\(^{2+}\)- and phospholipid-dependent protein kinase C (PKC) exists (as a single enzyme or possibly a family of related enzymes) in rodent \(\beta\)-cells. PKC activators probably induce secretion primarily through phosphorylation of key proteins, thereby sensitizing the exocytotic apparatus to Ca\(^{2+}\). PKC can be activated by several pharmacologic probes and by endogenous diacylglycerol (and possibly arachidonic acid) released by nutrient-activated phospholipases. Several nonspecific pharmacologic agents inhibit both PKC and physiologic insulin release. However, when a more specific inhibitor of PKC, H\(_7\) [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], was studied, it did not reduce glucose-induced insulin secretion. Moreover, prolonged preexposure of \(\beta\)-cells to a phorbol ester (believed to induce selective depletion of PKC) also failed to substantially reduce the subsequent secretory response to glucose. Thus, indisputable evidence for an obligatory physiological role of PKC in the islet is still missing, and the enzyme's status as a critical coupling signal should be viewed as putative only. *Diabetes* 37:3-7, 1988

CHARACTERISTICS OF ISLET PKC: HOW GOOD ARE AVAILABLE PROBES?

The characteristics of islet PKC are summarized in Table 1. It seems reasonably well established that there exists in the islet an enzyme (or possibly a family of enzymes) with properties of PKC; i.e., an enzyme that depends on Ca\(^{2+}\) and phospholipids and that selectively phosphorylates certain endogenous substrates (Fig. 1). In many cells, and presumably in the islet, activators of the cytosolic PKC lead to its translocation to the plasma membrane, where it may be proteolytically cleaved to a smaller-molecular-weight protein kinase M, which is biologically active even in the absence of Ca\(^{2+}\) (Fig. 1). It is likely that PKC resides in \(\beta\)-cells, as judged by the presence of PKC-like enzymatic activity and/or an insulinotropic response to known PKC activators in insulinoma cells or transformed \(\beta\)-cells (1-4). Diacylglycerol (DG) is one endogenous activator of PKC; recent indirect studies suggest that arachidonic acid (AA) may be another.

Because DG is rapidly metabolized, PKC is usually probed pharmacologically with phorbol esters, e.g., 12-O-tetradecanoylphorbol-13-acetate (TPA); nonphorbol but structurally related tumor-promoting diterpenes, e.g., mezerein; or structurally distinct tumor promoters, e.g., teleocidin. These compounds meet certain criteria required of selective PKC activators. For example, a phorbol ester that does not activate PKC (4a-phorbol-12,13-didecanoate) does not augment insulin release (unpublished observations). In addition, the effects of TPA or mezerein are appropriately inhibited by antimycin A (5), which depletes the islet of ATP; trifluoperazine (TFP), which inhibits PKC at concentrations higher than those required to inhibit calmodulin-dependent processes; or polymyxin B (6). The most specific inhibitor of PKC that is commercially available is H\(_7\) [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine] (7), which competes with ATP for binding to the enzyme (Fig. 1). This agent inhibits (albeit not completely) the effects of TPA on insulin release (Table 2). In addition, prolonged exposure to activators of PKC has been shown to lead to a selective loss of PKC activity in many cells, probably due to further proteolytic degradation of the enzyme after its translocation to the plasma membrane.

From the Division of Clinical Pharmacology, University of Colorado Health Sciences Center, and the Research Service, Denver Veterans Administration Medical Center, Denver, Colorado.

Address correspondence and reprint requests to Dr. Stewart A. Metz, Division of Clinical Pharmacology (C-237), University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Denver, CO 80262.

Received for publication 10 September 1987 and accepted in revised form 25 September 1987.
<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Properties of islet protein kinase C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous activators</strong></td>
<td></td>
</tr>
<tr>
<td>Ca⁺⁺, phospholipid (phosphatidylserine)</td>
<td></td>
</tr>
<tr>
<td>ATP ( (K_\text{m} \sim 10 \mu M) )</td>
<td></td>
</tr>
<tr>
<td>Diacylglycerol or arachidonic acid*</td>
<td></td>
</tr>
<tr>
<td><strong>Exogenous activators</strong></td>
<td></td>
</tr>
<tr>
<td>Phorbol esters (TPA)</td>
<td></td>
</tr>
<tr>
<td>Mezerein</td>
<td></td>
</tr>
<tr>
<td>1-Oleoyl-2-acetylglycerol</td>
<td></td>
</tr>
<tr>
<td>Exogenous phospholipase C*</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid*</td>
<td></td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>H 7 [1-(5-isouquinolinylsulfonyl)-2-methylpiperazine]</td>
<td></td>
</tr>
<tr>
<td>Cationic amphiphiles</td>
<td></td>
</tr>
<tr>
<td>Polymyxin B</td>
<td></td>
</tr>
<tr>
<td>Trifluoperazine (&gt;25-40 \mu M)</td>
<td></td>
</tr>
<tr>
<td>Melittin</td>
<td></td>
</tr>
<tr>
<td>Dibucaine</td>
<td></td>
</tr>
<tr>
<td>Antimycin A</td>
<td></td>
</tr>
<tr>
<td>Bromphenacetyl bromide (lipoxgenase inhibitors)</td>
<td></td>
</tr>
<tr>
<td>Dantrolene*</td>
<td></td>
</tr>
<tr>
<td><strong>Mechanism(s) of action</strong></td>
<td></td>
</tr>
<tr>
<td>Sensitizes exocytotic process to Ca⁺⁺</td>
<td></td>
</tr>
<tr>
<td>Increases cAMP*</td>
<td></td>
</tr>
<tr>
<td>Augments Ca⁺⁺ mobilization/electrical activity*</td>
<td></td>
</tr>
<tr>
<td>Activates phospholipase A₂*</td>
<td></td>
</tr>
</tbody>
</table>

TPA, 12-O-tetradecanoylphorbol-13-acetate.

*See text.

Is arachidonic acid an endogenous activator of PKC?

Activation of PKC by AA (or its metabolites) has been demonstrated in many types of nonendocrine cells (e.g., 15–18). Several indirect observations suggest that the same formulation may hold for the islet. Exogenous AA does stimulate Ca⁺⁺ mobilization in islets (19,20) and increases cytosolic free-Ca⁺⁺ levels (21) at concentrations that also promote insulin release (20,21). Thus, some effects of AA seem to depend on Ca⁺⁺ mobilization. However, AA also continues to elicit progressively greater insulin release at concentrations at which Ca⁺⁺ mobilization (as assessed by ⁴⁰Ca efflux) seems to reach a plateau; it also stimulates secretion in digitonin-permeabilized islets in which ambient Ca⁺⁺ concentrations are fixed by Ca⁺⁺-EGTA buffers (unpublished observations). Thus, some of the effects of AA seem to be independent of Ca⁺⁺ fluxes. AA-induced insulin release is vitiolated by concentrations of TFP that inhibit PKC activity (2), and it is reduced also by prolonged pretreatment of islets with TPA to deplete the islet of PKC activity (unpublished observations). Insulin release is also abrogated by dantrolene. This probe selectively blocks the effects of TPA or mezerein even in the absence of effects on Ca⁺⁺ mobilization, suggesting that dantrolene might be acting as a PKC inhibitor (unpublished observations). Furthermore, AA (but not oleic acid) actually inhibits TPA-induced insulin release (22); this finding may indicate that AA (like several other ligands for PKC) displaces TPA from its cellular receptor PKC (23,24). Together these data, although indirect, suggest that...
CAUSE Glucose stimulation of islets may elevate levels of AA might also have effects as a second endogenous activator of PKC in the islet. If true, this may be important because TPA is a relatively specific activator of PKC, certain findings derived from the use of that probe shed light on how PKC, if activated by physiologic agonists, might activate PLC, as judged by a rise in phosphatidylinositol turnover (21, 22), could contribute to the insulinotropic effects of PKC. This possibility merits further investigation.

Other studies suggest, however, that the major activity of PKC is to sensitize the exocytotic apparatus to a given level of Ca^{2+}, which is presumably mediated via the phosphorylation of key proteins on the secretory granule or in the cytoskeleton (Fig. 1). Thus, TPA provokes secretion in permeabilized islets bathed with a fixed Ca^{2+} concentration (28, 32) and reduces the EC_{50} for Ca^{2+}-induced secretion (33). It is tempting to conclude that physiologic agonists activate PLC and/or PLAr, either of which could yield a Ca^{2+}-mobilizing moiety [i.e., myo-inositol-1,4,5-trisphosphate (IP_3) or lysophospholipid, respectively] and a moiety that activates PKC and potentiates Ca^{2+}-dependent secretion (i.e., DG or AA, respectively), thereby eliciting maximal insulin release. Indeed, the Ca^{2+} and PKC limbs of secretion in intact islets seem to work together synergistically, which may explain the biphasic nature of nutrient-induced insulin release (34). Inhibitors of the lipoxygenase pathway for the metabolism of AA reduce the effects of TPA (13); it has thus been suggested that an intact islet lipoxygenase may modulate this effect of PKC to increase the Ca^{2+} sensitivity of the exocytotic apparatus (13).

Note that in many cells, pretreatment of cells with PKC activators even for only short periods elicits apparent negative (feedback) effects. The existence and importance of such inhibitory effects of PKC in the islet remain largely unexplored.

**TABLE 2**

Effects of H 7 or prolonged treatment with phorbol ester on insulin release induced by glucose or TPA

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 (H 7)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.7 mM glucose</td>
<td>3</td>
<td>72 ± 9</td>
</tr>
<tr>
<td>10 mM glucose</td>
<td>5</td>
<td>594 ± 40</td>
</tr>
<tr>
<td>10 mM glucose + 150 μM H 7</td>
<td>4</td>
<td>840 ± 78</td>
</tr>
<tr>
<td>16.7 mM glucose</td>
<td>5</td>
<td>1253 ± 55</td>
</tr>
<tr>
<td>16.7 mM glucose + 150 μM H 7</td>
<td>4</td>
<td>1638 ± 97</td>
</tr>
</tbody>
</table>

Stat 30-min incubations of intact rat islets were carried out as previously described (13). Data are μU insulin · 10^5 islets^{-1} · 30 min^{-1} (means ± SE). H 7, 1-(5-isoquinolinylsulfonyl)-2methylpiperazine; TPA, 12-O-tetradecanoylphorbol-13-acetate.

*H 7 (7) was present during preincubation and incubation. Such treatment had no effect on basal insulin release. H 7 (150 μM) inhibits TPA (2 μM)-induced secretion by 61 ± 7% (P < 0.01). Islets were pretreated with 1 μM TPA or dimethyl sulfoxide control for 18 h in RPMI-1640 medium containing 10% fetal calf serum. Islets then were washed and aliquoted into individual tubes in 16.7 mM glucose in the absence of TPA and preincubated 40 min in 1.7 mM glucose (total time between removal of TPA and start of incubation period was 2–2.5 h). Subsequent 30-min incubations were carried out. Values are from 3 experiments yielding similar results.

AA might also have effects as a second endogenous activator of PKC in the islet. If true, this may be important because glucose stimulation of islets may elevate levels of unesterified AA (19) well into the range needed to evoke insulin release (21).

**HOW DOES ACTIVATION OF PKC PROMOTE INSULIN RELEASE?**

With the premise that TPA is a relatively specific activator of PKC, certain findings derived from the use of that probe shed light on how PKC, if activated by physiologic agonists, might promote insulin secretion (Table 1). Several reported effects of TPA probably can be excluded as major contributors to the associated insulin release. TPA does augment islet cAMP levels (5), possibly in part by promoting glucagon release (25). However, this is unlikely to be a pivotal effect because TPA increases secretion at subthreshold and even subpotentiating glucose concentrations (5, 13), in contradistinction to agonists, which act purely by augmenting cyclic AMP levels. TPA does provoke a modest efflux of ^{45}Ca from prelabeled islets (unpublished observations and ref. 5) and may augment islet electrical activity (26). However, the increase in Ca^{2+} efflux may represent only the stimulation of Ca^{2+}-extrusion mechanisms (as described in other cell types); thus, note that Arkhammar et al. (2), in experiments with rat insulinoma cells, found that phorbol ester can augment insulin release even while depressing cytosolic free-Ca^{2+} concentrations. Also, Di Virgilio et al. (27) have observed that TPA may actually inhibit voltage-activated Ca^{2+} channels. Furthermore, TPA-induced insulin release does not require stimulation of Ca^{2+} uptake and is not blocked by D 600, a Ca^{2+}-channel blocker (5). Mobilization of intracellular Ca^{2+} also seems unimportant because TPA does not reduce cytosolic Ca^{2+} concentrations to be effective (2, 28). Effects of PKC on K^+ channels are probably irrelevant to TPA effects because TPA does not reduce 42Rb conductance (5). Furthermore, the effects of PKC on insulin release are additive to those of hypoglycemic sulfonamides (29), which probably act mainly by closing ATP-sensitive K^+ channels.

In many cells, possibly including the islet (30), PKC activators increase phospholipase A_2 (PLAr) activity (31). PKC may phosphorylate (and thereby inactivate) the PLAr inhibitory protein lipocortin. Conversely, the effect of TPA is inhibited by the PLAr inhibitor bremophenacyl bromide (13) at concentrations (~25 μM) fairly selective for the inhibition of PLAr. It cannot be excluded that bremophenacyl bromide could directly alkylate residues on PKC, i.e., without the intermediacy of PLAr. However, the effects of TPA are also inhibited by the removal of extracellular Ca^{2+} (5), a maneuver that also inhibits PLAr. Thus, products of PLAr activation, e.g., AA or lysophospholipids (21, 22), could contribute to the insulinotropic effects of PKC. This possibility merits further investigation.

With the premise that TPA is a relatively specific activator of PKC, certain findings derived from the use of that probe shed light on how PKC, if activated by physiologic agonists, might promote insulin secretion (Table 1). Several reported effects of TPA probably can be excluded as major contributors to the associated insulin release. TPA does augment islet cAMP levels (5), possibly in part by promoting glucagon release (25). However, this is unlikely to be a pivotal effect because TPA increases secretion at subthreshold and even subpotentiating glucose concentrations (5, 13), in contradistinction to agonists, which act purely by augmenting cyclic AMP levels. TPA does provoke a modest efflux of ^{45}Ca from prelabeled islets (unpublished observations and ref. 5) and may augment islet electrical activity (26). However, the increase in Ca^{2+} efflux may represent only the stimulation of Ca^{2+}-extrusion mechanisms (as described in other cell types); thus, note that Arkhammar et al. (2), in experiments with rat insulinoma cells, found that phorbol ester can augment insulin release even while depressing cytosolic free-Ca^{2+} concentrations. Also, Di Virgilio et al. (27) have observed that TPA may actually inhibit voltage-activated Ca^{2+} channels. Furthermore, TPA-induced insulin release does not require stimulation of Ca^{2+} uptake and is not blocked by D 600, a Ca^{2+}-channel blocker (5). Mobilization of intracellular Ca^{2+} also seems unimportant because TPA does not reduce cytosolic Ca^{2+} concentrations to be effective (2, 28). Effects of PKC on K^+ channels are probably irrelevant to TPA effects because TPA does not reduce 42Rb conductance (5). Furthermore, the effects of PKC on insulin release are additive to those of hypoglycemic sulfonamides (29), which probably act mainly by closing ATP-sensitive K^+ channels.

In many cells, possibly including the islet (30), PKC activators increase phospholipase A_2 (PLAr) activity (31). PKC may phosphorylate (and thereby inactivate) the PLAr inhibitory protein lipocortin. Conversely, the effect of TPA is inhibited by the PLAr inhibitor bremophenacyl bromide (13) at concentrations (~25 μM) fairly selective for the inhibition of PLAr. It cannot be excluded that bremophenacyl bromide could directly alkylate residues on PKC, i.e., without the intermediacy of PLAr. However, the effects of TPA are also inhibited by the removal of extracellular Ca^{2+} (5), a maneuver that also inhibits PLAr. Thus, products of PLAr activation, e.g., AA or lysophospholipids (21, 22), could contribute to the insulinotropic effects of PKC. This possibility merits further investigation.

Other studies suggest, however, that the major activity of PKC is to sensitize the exocytotic apparatus to a given level of Ca^{2+}, which is presumably mediated via the phosphorylation of key proteins on the secretory granule or in the cytoskeleton (Fig. 1). Thus, TPA provokes secretion in permeabilized islets bathed with a fixed Ca^{2+} concentration (28, 32) and reduces the EC_{50} for Ca^{2+}-induced secretion (33). It is tempting to conclude that physiologic agonists activate PLC and/or PLAr, either of which could yield a Ca^{2+}-mobilizing moiety [i.e., myo-inositol-1,4,5-trisphosphate (IP_3) or lysophospholipid, respectively] and a moiety that activates PKC and potentiates Ca^{2+}-dependent secretion (i.e., DG or AA, respectively), thereby eliciting maximal insulin release. Indeed, the Ca^{2+} and PKC limbs of secretion in intact islets seem to work together synergistically, which may explain the biphasic nature of nutrient-induced insulin release (34). Inhibitors of the lipoxygenase pathway for the metabolism of AA reduce the effects of TPA (13); it has thus been suggested that an intact islet lipoxygenase may modulate this effect of PKC to increase the Ca^{2+} sensitivity of the exocytotic apparatus (13).

Note that in many cells, pretreatment of cells with PKC activators even for only short periods elicits apparent negative (feedback) effects. The existence and importance of such inhibitory effects of PKC in the islet remain largely unexplored.

**DO PHYSIOLOGIC AGONISTS ACTIVATE PKC?**

The major source of DG, an endogenous activator of PKC, is the hydrolysis of phospholipids by PLC. Such an enzyme (or enzymes) exists in the islet and is almost certainly activated by carbachol or the rise in cytosolic free-Ca^{2+} concentration evoked by depolarizing concentrations of K^+ or by ionomycin (35). High glucose concentrations may also activate PLC, as judged by a rise in inositol phos-
phosphates IP$_3$ and myo-inositol-1,3,4,5-tetrakisphosphate (IP$_4$); 35]. The implication from such studies is that DG is also generated by glucose stimulation of islets, although this has rarely been directly assessed. For example, note that glucose-induced IP$_3$ or IP$_4$ generation may be considerably less than that evoked by the pharmacological stimuli (35); even this relatively small effect of glucose might be explained, at least in part, by effects on the metabolism of IP$_3$, induced by glucose or its metabolites (36) or by changes in cytosolic free-Ca$^{2+}$ concentration induced by fuels (35). It is reassuring, therefore, that Montague and Parkin (37) and Dunlop and Larkins (38) have provided data that glucose can elicit the production of DG. Dunlop and Larkins also provided evidence that glucose increases the phosphorylation of similar substrates as those affected by TPA. Thus, glucose may activate PKC or a related enzyme, but detailed studies of the relationship of that event to insulin release are needed.

**DOES SELECTIVE INHIBITION OR DEPLETION OF PKC IMPEDE PHYSIOLOGIC INSULIN RELEASE?**

The missing link is the absence of published evidence that a selective inhibition of PKC impedes physiologic insulin release. Clearly, polymyxin B (6), TFP, or antimycin A impedes glucose-induced insulin release, but each lacks specificity in that regard. I observed that H 7, the most specific PKC inhibitable (7), does not inhibit and actually modestly potentiates submaximal and maximal glucose-induced insulin release, even though it markedly reduces TPA-induced release (Table 2). Niki et al. (39) have also presented evidence in preliminary form that concentrations of H 7 that block TPA-induced insulin release do not impede that induced by glucose. Analogous data on H 7 in neutrophils have dissociated TPA-induced cell activation from the activation induced by other agonists (40,41). Furthermore, in the islet the polyamines putrescine and spermidine inhibit PKC (42); however, they do not block the effects of glucose on insulin release (42). As indicated above, prolonged exposure of cells to phorbol esters selectively depletes the cell of PKC (6–12). In recent experiments, I incubated islets for 18 h with 1 μM TPA, washed the cells, aliquoted them into individual test tubes, and assessed their ability to release insulin. Basal insulin release rates were not significantly altered by prior TPA pretreatment. However, the subsequent effect of TPA (2 μM) was obliterated by this maneuver, whereas glucose-induced secretion was not reduced (Table 2). (In fact, in 2 of 3 experiments, the effect of glucose was somewhat potentiated, as in studies of the effect of H 7.) Similar data have been reported with regard to the downregulation of PKC in pituitary cells and its lack of effect on their subsequent responsiveness to gonadotropin-releasing hormone (43).

**CONCLUSIONS**

A role for PKC as a modulator of the potentiating function of Ca$^{2+}$ in β-cells is a logical formulation. It does seem likely that glucose activates one or more PKC-like enzymes by the generation of DG, or possibly AA, and that certain pharmacologic probes stimulate insulin release, at least in part, by activating PKC. However, it has not been unequivocally demonstrated that PKC plays an obligate role in glucose-induced or other physiologic types of insulin release.

The failure of selective PKC inhibition or depletion to inhibit certain types of physiologic insulin release does not categorically exclude a role for PKC in the normal sequence of exocytosis. The β-cell may have multiplicative mechanisms to maintain relatively normal rates of secretion even if one modulatory pathway is defective or may function relatively normally if PKC is only partially inhibited (cf. ref. 12). Furthermore, the resistance of glucose-induced insulin release to inhibition by H 7 or some polyamines might be explained by the increments in intracellular ATP or Ca$^{2+}$ levels induced by glucose, because H 7 attacks the ATP-binding site on PKC (7), and polyamines compete with Ca$^{2+}$ in their interaction with PKC (42). In light of the molecular diversity of PKC forms described in some cells, it is possible that pretreatment with TPA downregulates only one of a family of PKC enzymes (or reduces the phosphorylation only of certain substrates) (cf. refs. 11 and 12), leaving others still able to be activated by glucose. Finally, a concomitant blockade of certain inhibitory effects of PKC by agents such as H 7 may obscure or offset the simultaneous inhibition of positive signals for insulin release expected of PKC blockers. Nonetheless, available data suggest that more study is needed to ascertain (or refute) that PKC has an obligate role in physiologic insulin secretion.

**ACKNOWLEDGMENTS**

The technical assistance of Mary Rabaglia and Doug Holmes is appreciated. This work was supported by a grant from the Veterans Administration and Public Health Service Grant RO1-DK-37312 from the National Institutes of Health. S.A.M. is a clinical investigator of the Veterans Administration.

**REFERENCES**