Dissociation of Protein Kinase C Redistribution from the Phosphorylation of Its Substrates*

(Received for publication, October 4, 1990)

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Increases in cytoplasmic [Ca\(^{2+}\)] caused by receptor activation are thought to stimulate the redistribution of loosely associated protein kinase C (PKC) to a tightly membrane-bound form that is activated by diacylglycerol. The precise role of Ca\(^{2+}\)-dependent redistribution of PKC in the activation of this enzyme has not been critically assessed. We examined the relationship between PKC redistribution and substrate phosphorylation by comparing the kinetics and the Ca\(^{2+}\) dependence of the two events. Using immunoblotting with specific PKC antibodies, we find that 1321N1 cells express the \(\alpha\) form of PKC, approximately 10–20% of which is membrane-associated in unstimulated cells. This fraction is increased to 60% in response to muscarinic receptor stimulation. Agonist-induced redistribution of PKC is rapid and transient, peaking at 30 s and returning to control levels by 2–5 min. Stimulation of muscarinic receptors also rapidly increases phosphorylation of both an endogenous 80-kDa protein and the peptide substrate, VRKRTLRLRL. However, unlike the time course of PKC redistribution, PKC-mediated phosphorylation of these substrates is sustained for up to 30 min. To compare the Ca\(^{2+}\) dependence of PKC redistribution and substrate phosphorylation, we buffered muscarinic receptor-induced increases in cytoplasmic [Ca\(^{2+}\)] with the divalent cation chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. Under these conditions, redistribution of PKC and phosphorylation of the exogenous peptide substrate are inhibited by about 80%. In contrast, muscarinic receptor-stimulated phosphorylation of the 80-kDa protein occurs even when increases in cytoplasmic [Ca\(^{2+}\)] are prevented. Taken together, these data demonstrate that the redistribution of PKC does not correlate in extent or duration with phosphorylation of PKC substrates.

Protein kinase C (PKC) is believed to regulate a variety of cellular functions including proliferation, secretion, and contractile events (for reviews, see Refs. 1 and 2). The signal transduction events that are involved in the activation of PKC and, thus, in the expression of such diverse cellular responses, are increases in cytoplasmic [Ca\(^{2+}\)] and the production of diacylglycerol (3). In vitro, the activity of this enzyme was found to strictly require Ca\(^{2+}\) and phospholipid, thus the designation Ca\(^{2+}\)- and phospholipid-dependent protein kinase (4). A specific role for Ca\(^{2+}\) in the activation of PKC was first suggested by the studies of Wolf et al. (5). Using inside-out erythrocyte vesicles and purified protein kinase, they found that physiological concentrations of Ca\(^{2+}\) cause the enzyme to bind to the membranes. An earlier finding that tumor-promoting phorbol esters stimulate the redistribution of PKC from an inactive, soluble form to an active, membrane-bound form suggested that the activity of PKC may be regulated by membrane association (6).

Recently, the idea that increases in intracellular [Ca\(^{2+}\)] stimulate the redistribution of PKC has been substantiated by studies examining the mechanism of PKC activation in intact cells. Treatment of GH4C1 rat pituitary cells with thyrotropin-releasing hormone results in a rapid shift of PKC activity from the soluble to the particulate cell fraction (7). When the cells are pretreated with Ca\(^{2+}\) ionophore, a manipulation shown to deplete releasable pools of intracellular Ca\(^{2+}\), thyrotropin-releasing hormone-stimulated subcellular redistribution of PKC is greatly inhibited. We demonstrated that muscarinic receptor-stimulated PKC redistribution is prevented when Ca\(^{2+}\) mobilization is inhibited in intact 1321N1 astrocytoma cells (8). In PC12 and bovine adrenal chromaffin cells, activation of nicotinic receptors causes an influx of extracellular Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels; the nicotinic receptor-mediated increase in membrane-associated PKC activity is substantially reduced when Ca\(^{2+}\) influx is prevented by channel blockers (9, 10). Additional evidence for the involvement of intracellular [Ca\(^{2+}\)] in PKC redistribution comes from studies using Ca\(^{2+}\) ionophores. In intact 1321N1 cells (8) and polymorphonuclear neutrophils (11), ionophore-induced elevation of intracellular [Ca\(^{2+}\)] is a sufficient signal for the redistribution of PKC.

Data obtained from mixed micelle assays indicate that PKC is active as a quaternary complex with Ca\(^{2+}\), phospholipid, and diacylglycerol (12). The model that has emerged from reconstitution and in vitro studies is that hormone-stimulated increases in cytoplasmic [Ca\(^{2+}\)] promote the association of soluble or loosely membrane-associated PKC with cell membranes where the enzyme encounters phospholipid cofactor, such as phosphatidyserine. In this state, PKC is thought to be inactive but sensitized for activation. Activation probably occurs when the enzyme binds diacylglycerol that is produced at cellular membranes in response to receptor-mediated hydrolysis of phospholipids (13). Inherent in this model is the notion that PKC redistribution to cell membranes is a prerequisite for enzyme activation. However, the precise relation-
ship between PKC redistribution and enzyme activation has not been directly examined.

We recently reported that [H]PDBu binding can be used to measure PKC redistribution in response to Ca^{2+}-mobilizing hormones in intact cells (8). Using this assay, we measure rapid and transient increases in [H]PDBu binding to intact 1321N1 cells stimulated with the muscarinic acetylcholine receptor agonist carbachol. We determined that the increase in [H]PDBu binding reflects an increase in phospholipid-esterifying binding sites at cell membranes, indicative of the redistribution of PKC from a soluble to a membrane-associated location. Muscarinic receptor-stimulated PKC redistribution mirrors the changes in cytosolic Ca^{2+} concentration. However, redistribution precedes any measurable increase in diacylglycerol mass, and, in fact, when diacylglycerol concentration is maximal, most of the PKC is no longer stably associated with cell membranes.

In the present studies, we use immunchemical localization of PKC in cell fractions to confirm that muscarinic receptor-mediated PKC redistribution is transient and dependent on increases in intracellular Ca^{2+}. Extending our previous findings, we address the temporal and quantitative relationship between the redistribution of PKC and phosphorylation of two specific PKC substrates. Through these studies, we seek to determine whether redistribution is, in fact, a good measure of PKC activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—We purchased [γ-^32P]ATP (3000 Ci/mmol), carrier-free P, [H]PDBu (12-19 Ci/mmol) from Du Pont. Mouse monoclonal antibody (clone MC5) directed against residues 312-323 of protein kinase C and known to recognize both the α and β isozymes of protein kinase C was from Amersham Corp. Protein kinase C isozyme-specific antibodies raised in rabbits against peptide sequences unique to the C-terminal regions of α, β, and γ protein kinase C (14, 15) were a generous gift of Dr. T. Saitoh (University of California at San Diego). Alkaline phosphatase-conjugated secondary antibodies, the corresponding substrate kit, and all reagents for electrophoresis were obtained from Bio-Rad. We obtained Amphotelin from Pharmacia LKB Biotechnology Inc. BAPTA/AM and fura-2/AM were purchased from Calbiochem. Peptide VRKRTLRRL was kindly provided by Dr. T. Hunter (Salk Institute, La Jolla, CA) and Dr. G. Johnson (National Jewish Center of Immunology, Denver, CO) and was purchased from Bachem, Inc. (Torrance, CA).

**Cell Culture**—1321N1 astrocytoma cells were grown as previously described (16). All studies were conducted on confluent populations of cells.

**Cell Lysis Fractionation**—For assessing expression of PKC isozymes, 1321N1 cells on 100-mm plates were rinsed with phosphate-buffered saline and lyed in 1.0 ml of a buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 5 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, and 0.01% leupeptin. After 15 min on ice, the cellular material was collected and sonicated at 12,000 × g for 5 min. The supernatant was either chromatographed on a DEAE-Sepacel column (17) or used directly for immunoblotting as described below. For detecting changes in the subcellular distribution of PKC in 1321N1 cells, fractions were prepared from cells treated for various times with carbachol, rinsed with ice-cold phosphate-buffered saline, and then broken by two cycles of freezethawing in buffer A (1 mM EGTA, 340 mM CaCl₂ (approximately 50 mM free Ca^{2+}), 50 mM β-glycerophosphate, 10 μg/ml leupeptin, and 0.3 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 37,000 × g for 10 min (4 °C), and the resultant supernatant was used as the soluble fraction. In some experiments, lysates were centrifuged at 100,000 × g for 60 min to confirm that PKC in the supernatant was not bound to a light vesicular membrane fraction. The pellet was reassembled and incubated on ice for 30 min in buffer A containing 0.5% Triton X-100 and no added CaCl₂. The membrane extract was then centrifuged at 37,000 × g for 30 min, and this supernatant was used as the particulate fraction.

**Immunoblotting of PKC**—Subcellular fractions or lysates were added to SDS sample buffer (18), and aliquots containing 200 μg of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide) and electrophoretically transferred to a nitrocellulose membrane. Nonspecific binding sites on the nitrocellulose were blocked by incubating the membrane with 3% nonfat milk for 18 h at 4°C. PKC was detected by blotting with isozyme-specific polyclonal antibodies (1:1000) or monoclonal anti-PKC antibodies (1:100, Amersham) followed by alkaline phosphatase-conjugated secondary antibodies as indicated. Blots were scanned with an LKB Ultrascan XL densitometer to quantify PKC immunoreactivity.

**Measurement of Cytoplasmic Ca^{2+} Concentration**—Cells were loaded with the Ca^{2+} indicator dye, fura-2/AM (1 μM), in the presence of 20 μM BAPTA/AM or MeSO vehicle for 30 min at 37°C. Changes in intracellular Ca^{2+} concentration were monitored as previously described (19).

**[H]PDBu Binding**—Measurements of equilibrium binding of [H]PDBu to intact 1321N1 cells were carried out as previously described (8).

**80-kDa Protein Phosphorylation**—After 18 h in serum-free medium, 1321N1 cells (approximately 10^5/35-mm plate) were rinsed twice with Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, 10 mM HEPES (pH 7.5), and 25 mM glucose) and incubated with this buffer containing 200 μCi/ml of carrier-free 32P. After approximately 2–4 h, cells were rinsed and exposed to various agents in Krebs buffer for given times. Cells were rinsed rapidly and scraped into 100 μl of boiling sampling buffer containing 0.5% SDS, 1.0% mercaptoethanol, 20 mM Tris-HCl (pH 8.0), 0.2 mM sodium orthovanadate, and 10 mM sodium fluoride. Samples were cooled, 50 μg/ml RNase A and 100 μg/ml DNase I were added, and then the samples were frozen on dry ice and lyophilized. Cellular proteins were reconstituted in 100 μl of electrophoresis sample buffer (9.5% urea, 2% Nonidet P-40, 5% β-mercaptoethanol, 1.6% pH 3.5-5 and 0.4% pH 3.5-10), and 10 μl was resolved by isoelectric focusing (approximately 10,000 V-hs) in the first dimension (20) and SDS-PAGE (10%) in the second dimension. The incorporation of 32P into the 80-kDa protein spots was quantitated by liquid scintillation counting of bands cut from gels.

**Peptide Phosphorylation**—Assays of VRKRTLRRL peptide phosphorylation in permeabilized cells were conducted at 37°C as described by Hesley and Johnson (21) except that 50 μg/ml saponin was used in place of digitonin and NaCl and KCl concentrations of the reaction buffer were changed to 20 and 115 mM, respectively.

**RESULTS**

Purification and molecular cloning of PKC have revealed that this enzyme exists as a family of at least seven isozymes (for reviews, see Refs. 22 and 23). Although structurally related, the PKC isoforms thus far identified, α, β, γ, δ, ε, and ζ, differ in tissue and cellular distribution (24-26) and, more subtly, in enzymatic properties (27-30). To better characterize the role of PKC in signal transduction, it is important to establish the identity of the isozymes expressed in the cell type being studied. Using PKC isozyme-specific antibodies in Western blotting analyses, we examined PKC expression in 1321N1 cell lysates. An immunoreactive 80-kDa band comigrated with purified PKC was detected with antibodies against PKC-α (Fig. 1). PKC-β and PKC-γ isozymes were undetectable in 1321N1 cells (lanes 3 and 5) even after cell lysates were enriched for PKC by DEAE-cellulose chromatography (31) (lanes 4 and 6).

Using [H]PDBu binding to intact 1321N1 cells, we measured a rapid and transient redistribution of PKC in response to muscarinic receptor stimulation (8). Here, we used immunoblotting with an anti-PKC antibody that recognizes the PKC-α isozyme to study the time course of carbachol-stimulated PKC redistribution. In unstimulated 1321N1 cells, approximately 10–20% of total immunoreactive PKC is associated with cell membranes, whereas the remainder partitions with the soluble cell fraction (Fig. 2A). Carbachol (500 μM) stimulates a rapid increase in membrane-associated PKC that is detectable by 15 s, the earliest time tested. Maximal association of PKC with cell membranes occurs after 30 s of muscarinic receptor stimulation, when approximately 50–60%
from lysates chromatographed on approximately 200 antibodies from a separate source (64) and similar results were obtained.

FIG. 1. PKC isozyme expression in 1321N1 cells. Approximately 200 μg of protein from whole cell lysates (lanes 1, 3, and 5) or from lysates chromatographed on a DEAE-cellulose column (lanes 2, 4, and 6) were subjected to 10% SDS-PAGE and transferred to nitrocellulose paper. Samples were immunoblotted with antipeptide antibodies specific for the α (lanes 1 and 2), β (lanes 3 and 4), and γ (lanes 5 and 6) PKC isozymes. In separate experiments, no immunoreactivity was detected using antibodies directed against the β isozyme. Immunoblotting was also conducted with isozyme-specific antibodies from a separate source (64) and similar results were obtained.

FIG. 2. Effect of carbachol on the subcellular distribution of PKC. A, confluent 1321N1 cultures were exposed to 500 μM carbachol for indicated times, and particulate and soluble fractions were prepared and analyzed for PKC immunoreactivity as described under "Experimental Procedures." The immunoreactive bands co-migrated with purified PKC at an apparent molecular mass of 80-kDa. B, PKC immunoreactivity in particulate fractions was quantitated by densitometry. Results are representative of data from three separate experiments.

of total cellular enzyme becomes membrane-associated. By 2–5 min of carbachol treatment, the cellular distribution of immunoreactive PKC is no longer different than that of control cells (Fig. 2B). An identical pattern of redistribution is seen when the soluble and membrane-associated fractions are defined using a high speed spin to bring down any PKC that might be associated with a light vesicular fraction. The time course of immunoreactive PKC redistribution confirms our findings from [3H]PDBu-binding experiments and suggests that the muscarinic receptor-stimulated redistribution of PKC from a soluble to a membrane-bound compartment is rapid but transient.

It is believed that the redistribution of PKC from the cytosol or from a loose membrane association to a stable membrane association primes the enzyme for activation (32). Therefore, PKC redistribution is very often used and accepted as a measure of enzyme activation. To examine the relationship between PKC redistribution and PKC activation, we compared the time course of muscarinic receptor-stimulated PKC substrate phosphorylation with the rapid and transient time course of PKC redistribution. As a measure of PKC activation, we chose the phosphorylation of an endogenous Mr 80,000 substrate. This protein, also referred to as the MARCKS protein (33) is identified as a major, specific substrate of PKC in many cells (34–36), including 1321N1 cells (37, 38). Using two-dimensional PAGE analysis of cellular extracts prepared from quiescent 1321N1 cells, we confirm the findings of Blackshear et al. (38) that carbachol stimulates the phosphorylation of the 80-kDa protein (Fig. 3A). We also find that the carbachol-stimulated increase in 80-kDa protein phosphorylation, like that stimulated by PMA, is markedly inhibited in cells rendered PKC-deficient by a 16-h exposure to 1 μM PMA (Fig. 3B). Under these conditions, PKC immunoreactivity is completely lost (39). These data confirm that the muscarinic receptor-mediated phosphorylation of the 80-kDa protein is a specific measure of PKC activation in 1321N1 cells.

The time course of 80-kDa protein phosphorylation induced by carbachol is shown in Fig. 4. An increase in 32P incorporation into the 80-kDa protein was detected as early as 30 s after carbachol addition; maximal 32P incorporation occurred after 2 min. In marked contrast to the transient, muscarinic receptor-induced redistribution of PKC, the phosphorylation of the 80-kDa protein by PKC remained sustained for at least 30 min after receptor occupation.

The sustained phosphorylation of the 80-kDa protein could be due either to the stability of the phosphate group attached to the substrate or to the continuous phosphorylation of this
substrate by PKC in response to receptor activation. To discriminate between these possibilities, we determined whether the time course of phosphorylation was affected by adding atropine to block muscarinic receptor activation during the sustained phase of carbachol-stimulated 80-kDa protein phosphorylation. Addition of 1 μM atropine to cells pretreated with carbachol for 5 min decreased the amount of 80-kDa phosphoprotein within 2.5 min (Fig. 4, inset). Similar results have been obtained from experiments using antagonists to bombesin and vasopressin receptors in Swiss 3T3 cells (40). That rapid dephosphorylation of the 80-kDa substrate occurs after muscarinic receptor blockade suggests that there are active phosphatases in the cells able to reverse PKC-mediated 80-kDa protein phosphorylation. These results suggest that it is not the stability of the phosphate group on the substrate but, rather, continuous activation of PKC that accounts for the sustained phosphorylation of the 80-kDa protein.

That the phosphorylation of a PKC substrate appears unrelated to enzyme redistribution may be unique to the 80-kDa protein. Therefore, we studied a second measure of PKC activity, namely the phosphorylation of an exogenous peptide substrate, VRKRTLRL. This peptide sequence is derived from the epidermal growth factor receptor and contains a PKC-specific phosphorylation site corresponding to threonine 654 (41, 42). Heasley and Johnson (21) introduced this peptide into permeabilized PC12 cells pretreated with growth factors and measured a PKC-dependent increase in peptide phosphorylation activity. In similar assays, we find that exposure of 1321N1 cells to carbachol stimulates VRKRTLRL peptide phosphorylation (Fig. 5). When PKC is down-regulated or when cells are treated with a pseudosubstrate peptide inhibitor of PKC (43), peptide phosphorylation is inhibited (data not shown). The carbachol-stimulated increase in VRKRTLRL phosphorylation is measurable as early as 1 min of carbachol treatment, is maximal by 2 min, and remains elevated above control phosphorylation for at least 10 min. Thus, in agreement with our data for 80-kDa substrate phosphorylation, VRKRTLRL peptide phosphorylation by PKC is also sustained longer than carbachol-stimulated redistribution of PKC.

To further explore the relationship between PKC redistribution and enzyme activation, we compared the Ca2+ dependence of the two events. Using the Ca2+ chelator, BAPTA, we first defined the conditions necessary to buffer hormonally stimulated increases in intracellular [Ca2+]. Within seconds, carbachol stimulates a transient increase in the cytoplasmic Ca2+ concentration from approximately 70 to 450 nM (Fig. 6, bottom). When cells have been loaded with 20 μM BAPTA/AM, the muscarinic receptor-induced increase in intracellular [Ca2+] is completely inhibited (Fig. 6, bottom).

The effect of BAPTA on the redistribution of PKC was investigated by two methods. Results from Scatchard analysis of equilibrium binding of [3H]PDBu to control and BAPTA-loaded 1321N1 cells are shown in Table I. As we have previously reported (8), carbachol stimulates a 2-3-fold increase in [3H]PDBu binding sites without significantly affecting the affinity of PKC for this ligand. This redistribution of PKC to the membrane, where it binds [3H]PDBu with high affinity, is greatly inhibited in BAPTA-loaded cells.

We obtained similar results when we measured the Ca2+ dependence of PKC redistribution by immunoblotting. Cells were incubated with vehicle or BAPTA for 30 min and then exposed to buffer or carbachol for 1 min. Soluble and particulate fractions were isolated, electrophoretically resolved, and assayed for immunoreactive PKC. As shown in Fig. 7A, the 3-fold increase in membrane-associated PKC that is stimulated by muscarinic receptor activation is inhibited by approximately 80% in cells loaded with BAPTA. Taken together with the results of the [3H]PDBu-binding experiments, these data indicate that muscarinic receptor-stimulated redistribution of PKC is dependent on increases in cytosolic [Ca2+].

Given that the redistribution of PKC is inhibited by preventing increases in cytoplasmic [Ca2+], we asked what effect this manipulation has on the presumed downstream activation of PKC. We used BAPTA to buffer increases in cytoplasmic [Ca2+] that stem from muscarinic receptor activation and measured the effect of [Ca2+] chelation on PKC-dependent phosphorylation. Surprisingly, we find no significant difference in carbachol-stimulated phosphorylation of the 80-kDa protein between control and BAPTA-loaded cells (Fig. 7B). Thus, whereas preventing increases in cytoplasmic [Ca2+] markedly attenuates muscarinic receptor-induced re-
Dissociation between PKC Redistribution and Activation

Fig. 6. Effect of BAPTA on carbachol-stimulated increases in intracellular Ca\(^{2+}\) concentration. Cells were loaded for 30 min with the fluorescent Ca\(^{2+}\) indicator fura-2/AM (1 \(\mu\)M) in the presence of BAPTA/AM (20 \(\mu\)M) or Me\(_2\)SO vehicle. Changes in intracellular [Ca\(^{2+}\)] were monitored fluorometrically, as described under "Experimental Procedures." The chelator had no effect on the basal cytoplasmic Ca\(^{2+}\) concentration. Carb, carbachol; Atr, atropine.

Table I

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>(K_C)</th>
<th>(B_{max})</th>
<th>-Fold</th>
</tr>
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<tbody>
<tr>
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<td>Control</td>
<td>30</td>
<td>800</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Carbachol</td>
<td>43</td>
<td>2550</td>
<td>3.2</td>
</tr>
<tr>
<td>B. BAPTA</td>
<td>Control</td>
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<td>660</td>
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<tr>
<td></td>
<td>Carbachol</td>
<td>30</td>
<td>1150</td>
<td>1.7</td>
</tr>
</tbody>
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distribution of PKC (Fig. 7A), it does not inhibit the phosphorylation of a prominent PKC substrate.

Finally, we tested the ability of carbachol to stimulate the phosphorylation of the VRKTRLRLR peptide substrate under conditions that block increases in intracellular [Ca\(^{2+}\)] and inhibit the redistribution of PKC. As shown in Fig. 7C, the 1.7-fold increase in substrate phosphorylation in response to carbachol is significantly inhibited in BAPTA-loaded cells. In contrast to 80-kDa protein phosphorylation, which is unaffected by the chelation of cytoplasmic Ca\(^{2+}\), the phosphorylation of the exogenous peptide substrate, VRKTRLRLR, appears to be dependent on Ca\(^{2+}\) mobilization and the concomitant redistribution of PKC.

Fig. 7. Effect of chelation of intracellular Ca\(^{2+}\) on carbachol-stimulated PKC redistribution, 80-kDa protein phosphorylation, and VRKTRLRLR peptide phosphorylation. A, 1321N1 cells were pretreated with BAPTA (20 \(\mu\)M) or Me\(_2\)SO vehicle for 30 min, washed, and exposed to buffer or carbachol (500 \(\mu\)M) for 1 min. Phosphorylation of the 80-kDa protein was then analyzed by two-dimensional gel electrophoresis as described under "Experimental Procedures." Data are expressed as fold-stimulation by carbachol and represent means \(\pm\) S.E. from four independent experiments. BAPTA did not affect 80-kDa protein phosphorylation in unstimulated cells. B, quiescent 1321N1 cells were labeled with \(^{32}\)P\_i for 2-4 h, pretreated with BAPTA (20 \(\mu\)M) or Me\(_2\)SO vehicle for the final 30 min, and exposed to buffer or carbachol (500 \(\mu\)M) for 1 min. Phosphorylation of the 80-kDa protein was then analyzed by two-dimensional gel electrophoresis as described under "Experimental Procedures." Data are expressed as fold-stimulation by carbachol and represent means \(\pm\) S.E. from four independent experiments. BAPTA did not affect 80-kDa protein phosphorylation in unstimulated cells. C, 1321N1 cells were pretreated for 30 min with BAPTA (20 \(\mu\)M) or Me\(_2\)SO vehicle. The cells were then rinsed, treated for 2 min with carbachol (500 \(\mu\)M), and assayed for peptide phosphorylation (see "Experimental Procedures" for details). Data have been corrected for phosphorylation in the absence of peptide and represent means \(\pm\) S.E. of triplicate samples from three independent experiments. Data are expressed as fold-stimulation by carbachol; BAPTA did not affect peptide phosphorylation in unstimulated cells.

Discussion

The objective of our investigation was to better understand the relationship between the redistribution of PKC and enzyme activation. We took two approaches to studying the
relationship between these two events in response to muscarinic receptor stimulation. First, we compared the time course of PKC redistribution measured by immunoblotting with that of PKC-dependent phosphorylation of both an endogenous 80-kDa protein and an exogenous peptide substrate. In a second approach, we related PKC redistribution to enzyme activation by studying the Ca$^{2+}$ dependence of the two hormone-stimulated events.

Phosphorylation of the acidic 80-kDa protein is increased in many intact cells stimulated with phorbol esters (35) or by hormones that activate PKC (38, 44, 45). The sites of phosphorylation of this protein in intact cells correspond well to the sites phosphorylated by purified PKC in vitro (46, 47). Furthermore, as shown here (Fig. 3) and by others (35, 44), receptor-stimulated phosphorylation of the 80-kDa protein is completely inhibited in cells rendered PKC-deficient by prolonged exposure to PMA. Thus, while it remains possible that the 80-kDa protein could be phosphorylated by other kinases (47), available evidence indicates that the 80-kDa protein phosphorylation is a good measure of PKC activation. The phosphorylation of the peptide VRKRTLRL also appears to be a specific measure of PKC activation since it is prevented by both PKC down-regulation and by a synthetic peptide inhibitor of PKC.

We report here that muscarinic receptor activation leads to a transient increase in membrane-bound PKC (Fig. 2). This finding confirms our results using [3H]PDBu binding to intact 1321N1 cells (8) and validates the use of PDBu binding in assaying PKC redistribution. Furthermore, these results are consistent with data from several laboratories depicting rapid and transient PKC redistribution elicited by a variety of hormones (7, 10, 48–50). Both the redistribution of PKC and PKC-mediated phosphorylation of substrates are maximal within 1 min of muscarinic receptor stimulation (Figs. 2, 4, and 5). Although a small amount of diacylglycerol produced concomitant with the hydrolysis of phosphatidylinositol bisphosphate may be adequate to support PKC activation, it is notable that we cannot measure an increase in diacylglycerol mass (8) or radiolabeled diacylglycerol (51) at this early time. We also demonstrate that PKC redistribution to cell membranes is transient, lasting only 2–5 min after hormone treatment, whereas diacylglycerol remains elevated (8, 39, 51) and phosphorylation of the 80-kDa protein and the peptide substrate VRKRTLRL is stimulated for up to 30 min.

Our finding that PKC-mediated phosphorylation of the 80-kDa protein and VRKRTLRL is sustained relative to PKC redistribution was unexpected. The sustained phosphorylation of the 80-kDa protein cannot be due to slow or inactive cellular phosphatases, because dephosphorylation occurs when muscarinic receptor stimulation is withdrawn (Fig. 4). Instead, the data suggest that PKC remains activated by receptor stimulation well beyond the period of PKC redistribution.

One explanation for the discrepancy between redistribution and substrate phosphorylation could be that PKC is initially activated by redistribution to cellular membranes but maintains activity in the absence of membrane association. Evidence that soluble PKC may retain catalytic activity stems from in vitro studies of PKC (52, 53) and studies of neutrophils stimulated with phorbol esters (54). These reports demonstrate that upon redistribution to cell membranes, PKC is proteolytically converted to an irreversibly active, soluble form that is independent of Ca$^{2+}$ and phospholipid. Several arguments can be made against proteolytic activation of protein kinase C occurring in 1321N1 cells stimulated with carbachol, however. First, the sum of soluble and particulate protein kinase C immunoreactivity remains constant throughout the time course of hormone-stimulated redistribution. Second, proteolytically activated protein kinase C should not require continuous receptor stimulation to be active. Third, we do not detect the production of either of the expected proteolytic fragments immunologically, using either monoclonal or PKC-α-specific antibodies, although at least the latter would recognize the catalytic domain of PKC. Moreover, it remains unclear whether proteolytically activated protein kinase C is physiologically relevant (2).

It must also be considered that 1321N1 cells might express PKC isozyme(s) not recognized by our antibodies, whose redistribution corresponds in time with the sustained phosphorylation events we have measured. However, the time course of PKC redistribution as assessed by [3H]PDBu binding to intact 1321N1 cells is as transient as that measured by immunoblotting (8). Considering that the phorbol ester-binding site is conserved among the known PKC isozymes, the increase in the amount of any PKC isozyme associated with membranes 2–5 min after agonist treatment must be very small.

Yet another explanation for the long term activation of PKC following apparently transient redistribution of the enzyme is that redistribution activates PKC, which in turn activates other protein kinases responsible for the sustained phosphorylation of substrates such as the 80-kDa protein. Activation of PKC by phorbol ester has been shown to stimulate ribosomal S6 protein kinase activity (55). In fact, in the 1321N1 cells, muscarinic receptor stimulation activates the S6 kinase and leads to the phosphorylation of the S6 protein secondary to activation of PKC (38). Activation of PKC also leads to the phosphorylation of c-rap and an increase in c-rap-associated kinase activity (56). To our knowledge, however, neither these nor any known protein kinases other than PKC have been shown to phosphorylate the 80-kDa protein.

A final possibility, and one that we consider most likely to account for the observed difference in the time courses of PKC redistribution and activation, is that a small fraction of the total PKC redistributed to cell membranes remains stably associated, whereas the rest resumes a loose membrane association. Activation of this small pool of PKC, perhaps together with PKC pre-existing at cellular membranes, could be supported by the increase in phosphatidylinositol-derived diacylglycerol that is sustained for at least 30 min during muscarinic receptor stimulation (39, 51). The amount of residual PKC that remains stably associated with cellular membranes, while virtually undetectable, may nonetheless be sufficient to catalyze complete phosphorylation of a preferred PKC substrate such as the 80-kDa protein.

In a second approach to studying the relationship between PKC redistribution and activation, we compared the Ca$^{2+}$ dependence of the two events. We demonstrate that muscarinic receptor-mediated redistribution of PKC is markedly inhibited when increases in intracellular Ca$^{2+}$ are buffered by loading cells with a Ca$^{2+}$ chelator (Fig. 7A). Using a similar experimental strategy, Fu et al. (57) and Martin et al. (50) reported that bradykinin- and thyrotropin-releasing hormone-stimulated Ca$^{2+}$ mobilization is essential to PKC redistribution in NCB-20 and GH$_3$ cells, respectively.

Since redistribution of PKC is believed to be a prerequisite for enzyme activation, increases in intracellular [Ca$^{2+}$] should regulate PKC activity. This relationship seems to apply in the case of phosphorylation of the exogenous peptide substrate, VRKRTLRL. Phosphorylation of this peptide is blocked under conditions that prevent increases in intracellular [Ca$^{2+}$] and PKC redistribution (Fig. 7C). Consistent
with these findings, muscarinic receptor-stimulated phosphorylation of at least one parietal cell PKC substrate is absolutely dependent on increases in intracellular Ca$^{2+}$ (58).

In contrast to the phosphorylation of the exogenous peptide substrate, 80-kDa protein phosphorylation by PKC is unaffected by BAPTA pretreatment, which inhibits PKC redistribution by 80% (Fig. 7B). One explanation for this finding is that the small amount of PKC (20%) that is redistributed even in the absence of increases in cytoplasmic [Ca$^{2+}$] may be sufficient to maximally phosphorylate the 80-kDa protein. A second possibility is that phosphorylation of this particular substrate is not dependent on enzyme redistribution at all. The observation that PKC can phosphorylate the nonmyristoylated form of the 80-kDa substrate, which is presumed not to associate with the membrane, implies that loosely associated or soluble PKC can catalyze phosphorylation of this substrate (59). Alternatively, muscarinic receptor stimulation of 1321N1 cells may result in the activation of that fraction of PKC that is membrane-associated even under resting conditions. In this case, diacylglycerol presumably produced from the rapid hydrolysis of phosphoinositides may activate the enzyme. In studies of vasopressin-stimulated hepatocytes (60) and platelets stimulated with platelet-activating factor (61), it was also concluded that only pre-existing membrane-associated PKC is activated. As argued above, there is also the possibility that the isozyme responsible for the phosphorylation of the 80-kDa protein in 1321N1 cells is not detected by our antibodies and has activity that is less sensitive to Ca$^{2+}$ (e.g. PKC$\alpha$, see Ref. 62) than PKC-$\alpha$.

Finally, the difference we observe between the 80-kDa protein and the peptide substrate with respect to the Ca$^{2+}$ dependence of their phosphorylation by PKC may reflect a substrate-specific Ca$^{2+}$ requirement. This could result from kinetic differences in the concentration or presentation of the two substrates (under the conditions used to assess phosphorylation. Of greater interest is the possibility that the phosphorylation by PKC of the peptide VRKRTLRLRL is indeed more dependent on Ca$^{2+}$ than that of the 80-kDa protein. This hypothesis is consistent with the finding that substrates impart cofactor requirements for PKC activity (63). If this is true in vivo, we may infer that redistribution of PKC and enzyme activation may also be related in a substrate-specific manner.

The data presented here clearly demonstrate that PKC substrate phosphorylation can occur or be sustained in the absence of significant increases in membrane-associated PKC. Consequently, PKC redistribution does not correlate in duration or extent with substrate phosphorylation. The relationship between PKC redistribution and PKC activation is complex and, as suggested by our data, may depend on the substrate in question. Therefore, conclusions regarding PKC activation ought to be based on direct measurements of substrate phosphorylation rather than inferred from measurements of PKC redistribution. Currently, there are only a few recognized PKC substrates; even fewer have known identity (2). Obviously, a thorough understanding of the precise relationship between PKC redistribution and activation will require further identification of PKC substrates. A relationship of PKC redistribution and activation that is contingent on substrate would allow for great diversity in both the regulation of PKC activation and in the cellular responses affected by this ubiquitous enzyme. Of course, the potential for diversity is further enhanced by the existence of multiple, different PKC isozymes.

Acknowledgments—We would like to thank I. Paul Shapiro for help with two dimensional gel electrophoresis. We are also grateful to David Goldstein and Paulina Quintana for masterful technical assistance.

REFERENCES

Dissociation between PKC Redistribution and Activation