Factors Influencing Chelator-stable, Detergent-extractable, Phorbol Diester-induced Membrane Association of Protein Kinase C

DIFFERENCES BETWEEN Ca2+-INDUCED AND PHORBOL ESTER-STABILIZED MEMBRANE BINDINGS OF PROTEIN KINASE C

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One of the early events associated with the treatment of cells by tumor promoter phorbol esters is the tight association of protein kinase C to the plasma membrane. To better understand the factors that regulate this process, phorbol ester-induced membrane binding of protein kinase C was studied using homogenates, as well as isolated membranes and purified enzyme. Addition of 12-O-tetradecanoylphorbol 13-acetate (TPA) to the homogenates of parietal yolk sac cells and NIH 3T3 cells in the presence of Ca2+ resulted in plasma membrane binding of protein kinase C which subsequently remained bound to the membrane independent of Ca2+. Although protein kinase C was activated by TPA in the absence of Ca2+ and by diolein in the presence of Ca2+, both these agents when added to homogenates under these respective conditions had no effect on membrane association of protein kinase C. However, under these conditions relatively weak binding of protein kinase C was found if purified protein kinase C was used with isolated membranes. Binding studies using purified protein kinase C and washed membranes showed that the binding of the TPA-kinase complex to membranes required phospholipids and reached saturation at 0.1 unit (24 ng of protein kinase C)/mg of parietal yolk sac cell membrane protein. Phorbol ester treatment of cells in media with and without Ca2+ showed that the TPA-induced increase in membrane-associated protein kinase C was regulated by Ca2+ levels even in intact cells. TPA-stabilized membrane binding of protein kinase C differs in several aspects from the previously reported Ca2+-induced reversible binding. TPA-stabilized binding of protein kinase C to isolated membranes is temperature dependent, relatively high in the plasma membrane-enriched fraction, saturable at physiological levels of protein kinase C, requires the presence of both membrane protein(s) and phospholipids, and further requires the addition of phospholipid micelles. In contrast, Ca2+-induced reversible binding is more rapid, not appreciably influenced by temperature, not selective for a particular subcellular fraction, not saturable with physiological amounts of protein kinase C, exhibits trypsin-insensitive membrane binding sites, and requires membrane phospholipids but not added phospholipid micelles.

Tumor promoter phorbol esters exert their effects by binding to specific receptors located primarily on cell surface membranes (for review see Ref. 1). Ca2+- and phospholipid-dependent protein kinase C is fully activated by biologically active phorbol esters such as TPA in the presence of Ca2+ and phospholipid (2). Tumor promoter TPA activates this kinase apparently by substituting for diacylglycerol, a physiological protein kinase C activator formed by the breakdown of phosphatidylinositol in a response to external stimuli (3-5). Protein kinase C can serve as a cellular receptor for phorbol ester and may account for most, if not all, of the specific phorbol ester binding (6, 7).

In an earlier study using EL4 thymoma cells and PYS cells, it has been shown that TPA treatment of these intact cells results in a dramatic decrease in protein kinase C activity in the cytosol accompanied by an increase in tightly membrane-bound protein kinase C activity (8, 9). This early change in protein kinase C in response to phorbol ester treatment of intact cells has been confirmed in several other cell types (10-13). Protein kinase C has been shown to phosphorylate several membrane-associated proteins that regulate growth and morphology including receptors for growth factors (epidermal growth factor, insulin, and somatomedin), vinculin, and transferrin receptor (14-19). Yet, protein kinase C is distributed predominantly in the cytosol of certain cell types and tissues (20-24). Since phorbol esters are believed to act primarily at the cell surface, the induced rearrangement, or stabilization, of protein kinase C activity to the plasma membrane seems to be an early event involved in the initiation of tumor promotion. The mechanism by which this kinase tightly associates to the plasma membrane is not understood.

Several possible mechanisms can be evoked to explain TPA-induced protein kinase C redistribution. Treatment of cells with phorbol esters induces production of reactive oxygen species, which are known to enhance lipid peroxidation and oxidative modification of certain amino acid residues in proteins (25). Lipid peroxidation in turn cross-links proteins and affects all aspects of cell organization, including membrane and surface structure (25). Alterations in plasma membrane and/or modification of protein kinase C by free radicals could affect the membrane association of protein kinase C. A second possibility is that increased intracellular Ca2+ may be initially involved in membrane association of protein kinase C as Ca2+ alone has been noted to modulate reversible protein kinase C

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol 13-acetate; EGF, [ethylenedioxy(oxymethylene)tritol]tertaraacetic acid; PS, phosphatidylserine; PD Bu, phosphol 12,13-dibutyrate; PYS, parietal yolk sac; DMEM, Dulbecco-Vogt modified Eagle's medium.
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binding to membranes (7, 23) which subsequently may be converted to a stable binding. A third possibility is hydrophobic interaction between the protein kinase C-phospholipid-TPA complex and the membrane, either in a specific or nonspecific manner (26). One or a combination of the above factors may be responsible for the rearrangement of this kinase to the membrane.

In this communication we identify factors that may be involved in regulating association of protein kinase C to membranes in cell homogenates, and the binding of purified enzyme to the isolated membranes, and also present evidence that TPA-induced binding of protein kinase C to the plasma membrane may be at specific saturable sites.

EXPERIMENTAL PROCEDURES

Materials—L-a-Phosphatidylserine, 1,2-diolein, histone (type III-S), leupeptin, phospholipase C from Clostridium perfringens (type XII, 600 units/mg protein), and phospholipase A2 from Naja naja mambica mambica (1570 units/mg protein) were obtained from Sigma. TPA and PDBu were purchased from Pharmacia P-L Biologicals. [1-14C]ATP (25 Ci/mmol) was from ICN Radiochemicals. Wistar female rats (2-3 months old) were used to obtain tissues. Rats were killed under ether anesthesia, and the tissues were immediately chilled in an ice bucket and used without delay.

Membrane Association of Protein Kinase C in Cell Homogenates—PYS-2 cells and NIH 3T3 cells were grown in 150-cm² culture flasks in Dulbecco-Vogt modified Eagle's medium (DMEM) containing 10% fetal calf serum. Confluent cells were used in all experiments. Cells were homogenized in buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 0.15 μM pepstatin A) at 4 °C with a Dounce homogenizer. To 2-ml aliquots of homogenate in screw-capped tubes were added TPA (0.1 μM) or phosphatidylserine (20 or 200 μg/ml) and diolein (0.8 or 8 μg/ml) as indicated to a final volume of 3 ml. After warming the reaction mixture to 25 °C, 20 μl of 0.1 M CaCl₂ was added (free Ca²⁺, ~150 μM) and the reaction tubes were incubated for 26 min at 25 °C with end-to-end rotation. Thirty μl of 0.1 M EGTA then was added to each tube, and the tubes were cooled in an ice bucket for 5 min and centrifuged at 15,000 × g for 15 min at 4 °C. The supernatant was retained and the pellet was resuspended with 5 ml of buffer A to wash and again centrifuged as above. The washed pellet was extracted with 3 ml of buffer A containing 1% Nonidet P-40 at 4 °C for 1 h and then centrifuged to separate detergent-solubilized material. Protein kinase C activity present in both the soluble fraction and detergent-solubilized particulate fractions was measured (27).

Binding of Partially Purified Protein Kinase C to Isolated Membranes—The crude membrane fraction isolated from cell homogenates by centrifugation at 15,000 × g was washed twice with buffer A to remove unassociated coreceptors from the membranes. These washed membranes were suspended in buffer A at a protein concentration (detergent-solubilized protein) of 0.5-1.0 mg/ml and divided into 3-ml aliquots. These washed membranes were suspended in 20 mM Tris-HCl, 0.15 M NaCl at a protein concentration of 1-2 mg/ml and divided into 3-ml aliquots. These samples were warmed to 25 °C and then treated with trypsin (50 μg/ml), phospholipase C (20 μg/ml), or phospholipase A2 (10 μg/ml) as indicated. After incubation the treated membranes were washed twice with buffer A and used in the determination of both Ca²⁺-induced and TPA-stabilized membrane bindings of protein kinase C.

Modification of Membranes by Trypsin and Phospholipases—The particulate fractions obtained from rat tissues or PYS cell homogenates by centrifugation at 13,000 × g were washed twice with 20 mM Tris-HCl, pH 7.5 (protease inhibitors omitted) and then twice with 20 mM Tris-HCl, 0.15 M NaCl (pH 7.5) and centrifuged. Finally the membranes were suspended in 20 mM Tris-HCl, 0.15 M NaCl at a protein concentration of 1-2 mg/ml and divided into 3-ml aliquots. These samples were warmed to 25 °C and then treated with trypsin (50 μg/ml), phospholipase C (20 μg/ml), or phospholipase A2 (10 μg/ml) as indicated. After incubation the treated membranes were washed twice with buffer A and used in the determination of both Ca²⁺-induced and TPA-stabilized membrane bindings of protein kinase C.

RESULTS AND DISCUSSION

There are at least two types of protein kinase C association with the cell membrane. One type, reversible Ca²⁺-induced membrane binding, can be dissociated by chelators (7, 23). A second type, TPA (0,1 μM)-induced3 membrane binding, is stable to chelators and high salt but can be dissociated with detergents (9). The TPA-induced membrane binding observed in the phorbol ester-treated cells is also stable even after

3 Increase in the membrane-associated protein kinase C activity noted with TPA treatment of cells can be interpreted as the TPA stabilization of binding of already loosely membrane-associated enzyme rather than a true translocation of the cytosolic protein kinase C (9). As shown in the latter part of this report, TPA stabilizes and modifies the binding of a certain fraction of the Ca²⁺-dependently bound protein kinase C to the particulate fraction. Since TPA also has been reported to increase the affinity of protein kinase C for Ca²⁺ (2, 6, 7), it may also induce the translocation of protein kinase C by lowering its Ca²⁺ requirement. Thus, both the terms “TPA-induced” and “TPA-stabilized” membrane binding have been used in the text.
omission of phorbol ester (TPA or PDBu) from the media used to wash the treated cells and from the buffer used to homogenize these cells and wash the membranes (9). While identifying factors that influence this chelator-stable TPA-induced protein kinase C membrane binding in homogenates, or with isolated membranes, the above criteria were followed by having the chelator EGTA present during membrane isolation to ensure that only tightly bound detergent-extractable protein kinase C activity was measured. Since TPA-induced binding of protein kinase C previously has been shown to occur predominantly at the plasma membrane (9), the particulate fraction sedimenting at 13,000 × g was used as a source of crude plasma membranes, and the soluble fraction thus obtained was referred to as cytosol. In subsequent experiments, the particulate fraction was further divided into nuclear-enriched (P1), mitochondria-plasma membrane-enriched (P2), and microsomal-enriched (P3) subfractions.

Factors Influencing TPA-induced Protein Kinase C Membrane Binding

In Cell Homogenates—Incubation of total PYS cell crude homogenates with TPA alone for 20 min resulted in a 50% decrease in protein kinase C activity in the soluble fraction, but no increase in protein kinase C activity in the membrane fraction. However, treatment of PYS homogenates with TPA in the presence of 0.1 mM CaCl₂ resulted in both a 95% decrease in protein kinase C activity in the cytosolic fraction and a pronounced increase in membrane-associated protein kinase C activity (Table I). About 55% of the activity lost from the soluble fraction was recovered in the particulate fraction. Although Ca²⁺ was required to initiate this TPA-induced membrane association, once protein kinase C was bound to the membrane the continued presence of Ca²⁺ and a pronounced increase in membrane-associated protein kinase C activity remained associated with membranes iso-

tratious. With NIH 3T3 cells protein kinase C activity can be measured directly in the cytosol without the need for prior purification by DEAE-cellulose chromatography. In this cell type as well, lipids added to crude homogenates had no influence on the membrane association of protein kinase C. Likewise superoxide radicals generated by the xanthine-xanthine oxidase system had no effect on protein kinase C membrane association.

Effect of TPA and diolein addition to homogenates on the chelator-stable membrane association of protein kinase C

To 2 ml of homogenate were added TPA (0.1 μM) or the indicated amounts of PS and diolein to a final volume of 3 ml. After warming the reaction mixture to 25 °C, 20 μl of 0.1 M CaCl₂ were added. The reaction mixture was incubated for 20 min at 25 °C and then chelator-stable detergent-extractable membrane-bound protein kinase C activity was measured.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>PYS cell protein kinase C activity*</th>
<th>NIH 3T3 cell protein kinase C activity*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Soluble Membrane bound</td>
<td>Soluble Membrane bound</td>
</tr>
<tr>
<td>Control</td>
<td>0.442 (100)</td>
<td>0.070</td>
</tr>
<tr>
<td>TPA</td>
<td>0.318 (72)</td>
<td>0.076 (66)</td>
</tr>
<tr>
<td>TPA, CaCl₂</td>
<td>0.021 (5)</td>
<td>0.030 (6)</td>
</tr>
<tr>
<td>PS (20 μg/ml), diolein (0.8 μg/ml)</td>
<td>0.456 (103)</td>
<td>0.074 (107)</td>
</tr>
<tr>
<td>PS (300 μg/ml), diolein (8 μg/ml), CaCl₂</td>
<td>0.438 (99)</td>
<td>0.069 (83)</td>
</tr>
</tbody>
</table>

* Protein kinase C activity is expressed in units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H₁/min at 30 °C.

It has been shown that TPA does not bind independently to protein kinase C but requires the presence of phospholipids (6, 7). Though the stable membrane association of protein kinase C occurs in homogenates with the addition of TPA and Ca²⁺ alone without the further addition of phospholipids, endogenous phospholipids present in the crude homogenate probably participate in this process. Though optimal binding of TPA to the protein kinase C phospholipid complex takes place in the presence of Ca²⁺, a significant amount of TPA binding and 50–70% of the maximal TPA plus Ca²⁺-stimulated enzyme activity can be obtained with TPA alone in the absence of added Ca²⁺ (6, 7). The TPA-induced membrane association of protein kinase C, however, is strictly Ca²⁺ dependent.

Though diolein activates protein kinase C in a manner similar to TPA, the addition of diolein to homogenate preparations either alone or in combination with PS had little apparent effect in stabilizing the membrane association of protein kinase C (Table I). Measurement of protein kinase C activity in PYS cells requires prior DEAE-cellulose chromatographic fractionation of the cytosol to remove endogenous components that interfere with the assay (20). Assuming the presence of similar substances that might interfere with the binding of these lipids with protein kinase C, or assuming the rapid metabolism breakdown of these added lipids, excess amounts of diolein and PS were added to the crude homogenate. Again, no diacylglycerol-phospholipid-Ca²⁺-mediated membrane association of protein kinase C could be demonstrated. With NIH 3T3 cells protein kinase C activity can be measured directly in the cytosol without the need for prior purification by DEAE-cellulose chromatography. In this cell type as well, lipids added to crude homogenates had no influence on the membrane association of protein kinase C. Likewise superoxide radicals generated by the xanthine-xanthine oxidase system had no effect on protein kinase C membrane association.

In Isolated Membranes—To better understand the factors that might regulate binding of protein kinase C to membranes, binding of partially purified protein kinase C to isolated membranes in the presence of various agents was studied. TPA alone in the presence of Ca²⁺ was not able to promote binding of protein kinase C to the membrane preparation (Table II). However, the further addition of PS to this reaction mixture resulted in the tight association of protein kinase C to the membrane. PS alone had no effect. In experiments carried out in the absence of PS, only 10–30% of protein kinase C activity remained associated with membranes isolated in the presence of EGTA. Even this low amount of membrane-bound protein kinase C activity was dissociated from the membrane fraction when washed with EGTA-containing TPA-free buffer. Although membrane phospholipids facilitate TPA binding with protein kinase C they may not be sufficient to promote membrane integration of protein kinase C. PS micelles may facilitate membrane integration of the

³ With addition of the regular amount of phospholipids (PS, 20 μg/ml and diolein, 0.5 μg/ml) to the protein kinase C assay mixture it is possible to demonstrate phospholipid-stimulated protein kinase C activity (at 50 μM Ca²⁺) in the crude extracts of NIH 3T3 cells. Since we added 10-fold the amount of these two lipids for membrane binding of protein kinase C, we assumed an excess. In another experiment these high amounts of lipids were added both at the beginning and also in the middle of the incubation (10 min) to ensure availability of these lipids. From these experiments it is likely that the observed lack of diolein to promote the membrane association of protein kinase C in the crude homogenates is unlikely due to its rapid depletion in the reaction since it was made available in adequate amounts.

bound Ca\(^{2+}\)-TPA-protein kinase C complex. Phosphatidylethanolamine and phosphatidylglycerol, respectively, were only 60 and 25% as effective when compared to PS in promoting protein kinase C association to membrane. Phosphatidylcholine was completely ineffective. This specificity in phospholipid requirement for protein kinase C membrane association is similar to that previously reported for protein kinase C activation and for TPA binding (7, 8). When EGTA was added to the mixture of TPA and PS, membrane binding of protein kinase C was decreased by 65% (Table II). Under conditions that were optimal for TPA-induced protein kinase C binding to isolated membranes, PDBu and diolein each promoted about 60% of stable binding relative to that amount of binding mediated by TPA. In the absence of membranes no sedimentation of protein kinase C occurred suggesting that the experimental conditions described do not favor the sedimentation of the kinase with lipid vesicles.

With TPA-induced binding, under the optimal conditions described above, saturation in membrane binding of protein kinase C occurred with a half-maximal binding at 1.2 units of enzyme/mg membrane protein (Fig. 1). Using a fixed amount of protein kinase C (10 units) and increasing membrane protein (2-12 mg) a linear increase in enzyme binding was observed (Fig. 2). Based on the specific activity of the purified homogeneous enzyme (33), the amount of specific protein kinase C bound to membrane was calculated and found to be approximately 24 ng/mg of membrane protein.\(^5\) Binding of such a limited amount of enzyme to the membrane suggests that this association may take place at specific receptor(s) in the membrane and not through nonspecific association of the protein kinase C-lipid complex.

While in intact cells only the inner surface of the plasma membrane is accessible for the binding of protein kinase C, with homogenates and isolated membranes the outer surface of the membrane also is accessible. However, under the experimental conditions described here the amount of protein kinase C bound per mg of membrane protein was approxi-

\(^{5}\) Highly purified homogeneous protein kinase C rapidly loses its activity (33). Hence most of this study was carried out using partially purified protein kinase C. It was assumed that the turnover number of protein kinase C is the same in the partially purified state as in the homogeneous state.
with the plasma membrane for binding of the protein kinase C complex formed under these latter two conditions. This may account for the finding in crude homogenates, as presented in Table I, that no binding of protein kinase C to the membrane is noted with addition of diolein plus Ca2+.

Incubation of the diolein-protein kinase C complex with membranes previously saturated with TPA-protein kinase C resulted in no further binding of protein kinase C, suggesting that both these regulators promote protein kinase C binding to the same membrane sites. Since the number of specific membrane sites may be limited, the presence of varying numbers of vacant binding sites on different cell membranes may explain why cell types may differ in the amount of cytosolic protein kinase C bound to membranes upon TPA treatment.6

In Intact Cells—To further elucidate the role of Ca2+ in modulating TPA-induced redistribution of protein kinase C, intact PYS cells in media with and without Ca2+ were treated with TPA. In both the presence and absence of extracellular Ca2+ a dramatic decrease in the activity of cytosolic protein kinase C occurred within 15 min (Fig. 3B). However, only in the cells treated with TPA in the Ca2+-containing medium did the particulate fraction exhibit an elevation in protein kinase C activity (Fig. 3A). In the cells which were initially treated with TPA in the absence of Ca2+ and showed no increase in particulate fraction protein kinase C activity, subsequent addition of Ca2+ to the treated cells resulted in an increase in membrane-bound protein kinase C activity.

In all experiments using either homogenates (Table I) or intact cells (Fig. 3), not more than 50% of the activity lost from the soluble fraction was recovered associated with the membrane fraction. However, treating the soluble fraction prepared following TPA treatment with the detergent Nonidet P-40 (1%) followed by DEAE-cellulose chromatography resulted in complete recovery of the missing activity which apparently remained in the cytosol in an inactive form. This initially unaccounted for activity was not found associated with microsomes sedimented at 100,000 x g, but was present in the 100,000 x g supernatant. Protein kinase C in the presence of TPA may bind to other protein(s) or hydrophobic components in the cytosol of homogenates and, therefore, not be eluted with 0.08 M NaCl from DEAE-cellulose. No particulate kinase C activity was detected, however, either in the unbound protein fraction or in the other fractions eluted from DEAE-cellulose with a 0.0–0.2 M NaCl gradient. Alternatively, the protein kinase C activity may be masked in these fractions by apparent TPA-mediated association with interfering components. The observation that as much as 50% of the TPA-modified protein kinase C activity can be recovered as detergent-solubilized activity from the cytosol suggests that in addition to acting at the membrane, phorbol esters also may exert their actions by modifying protein kinase C interactions with cytosolic components, particularly in the absence of extracellular Ca2+.

6 Assuming the water insolubility of diolein is responsible for the observed differences in diolein action compared to TPA in the crude homogenates and in the membranes, we used the more soluble analogue, l-oleoyl 2-acetylglycerol. However, it was found to have little effect in the crude homogenates and produced either the same or slightly less protein kinase C binding as compared to diolein under the same experimental conditions as described in Table II.

The amount of cytosolic protein kinase C activity that associated with membranes after TPA treatment varied in different cell types. In PYS and NIH 3T3 cells it was about 30–50% whereas in B16 melanoma cells it was more than 90%. Membrane association of only 20% of the cytosolic activity of protein kinase C in TPA-treated leukemia cells was reported (42).
FIG. 4. Effect of temperature on Ca2+-induced and TPA-stabilized membrane bindings of protein kinase C (C-kinase). To determine TPA-induced chelator-stable binding (•, □) PYS membranes (5 mg of protein) were incubated for the indicated periods of time at 4 °C (○, ◇) or 25 °C (●, ▲) in the presence of partially purified protein kinase C (10 units), TPA (0.1 μM), PS (20 μg/ml), 0.1 mM Ca2+, and 20 μM leupeptin in a total volume of 3 ml. EGTA (1 mM final) was added to each incubation sample to terminate the binding reaction, and membranes were immediately isolated by centrifugation. The membranes were washed once with 10 ml of chelator-containing buffer A, and Ca2+-induced reversibly bound protein kinase C binding (●, ▲) was studied in the same manner except that TPA and PS were omitted during incubation. At the indicated times, membranes were rapidly isolated by centrifugation, washed once with 10 ml of Ca2+-containing buffer A, and Ca2+-induced reversibly bound protein kinase C was extracted with 1 mM EGTA.

As shown in Table III, the cytosolic fractions isolated in the presence of Ca2+ contain 60-100% lower activity when compared to cytosolic fractions isolated in the presence of EGTA. Most of this lost cytosolic activity can be recovered by chelator treatment of the particulate fractions and appears to be accounted for by Ca2+-induced reversible binding to fractions enriched in nuclear (P1), plasma membrane plus mitochondrial (P2), and microsomal components (P3). The relative amounts of Ca2+-induced protein kinase C binding to these subcellular fractions varied with the tissue source. For example, in the heart and kidney, the nuclear-enriched fraction contained the most protein kinase C followed by the plasma membrane- and microsomal-enriched fractions, whereas in the spleen the microsomal-enriched fraction bound higher protein kinase C activity. In brain both microsomal- and nuclear-enriched fractions exhibited the same amount of Ca2+-induced protein kinase C binding. In the subcellular fractions isolated from these different tissues, the total

![Graph showing effect of temperature on Ca2+-induced and TPA-stabilized membrane bindings of protein kinase C (C-kinase).](image)

**TABLE III**

| Tissue | Cytosol EGTA Ca2+ P1 nuclear P2 plasma membrane and mitochondrial P3 microsomal P1 nuclear P2 plasma membrane and mitochondrial P3 microsomal | TPA-stabilized binding units/g wet tissue
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</thead>
<tbody>
<tr>
<td>Heart</td>
<td>10.0 2.0 (80)°</td>
<td>4.43 (56)°</td>
<td>1.50 (13)</td>
<td>0.86 (11)</td>
<td>0.35 (6)</td>
<td>0.24 (23)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>1.82 0.7 (62)</td>
<td>1.84 (164)</td>
<td>0.85 (76)</td>
<td>0.13 (12)</td>
<td>0.28 (15)</td>
<td>0.41 (48)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>12.88 0 (100)</td>
<td>3.36 (26)</td>
<td>2.36 (18)</td>
<td>8.2 (64)</td>
<td>0.84 (25)</td>
<td>2.15 (91)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>27.5 9.2 (67)</td>
<td>6.68 (38)</td>
<td>5.42 (30)</td>
<td>6.88 (38)</td>
<td>0.49 (7)</td>
<td>2.33 (38)</td>
<td>0</td>
<td></td>
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</tbody>
</table>

° Protein kinase C activity is expressed in units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H1/min at 30 °C.
° The values in parentheses represent the percentage of the activity decreased in the cytosol (Ca2+ treated) compared to control conditions (EGTA treated).
° EGTA-extractable Ca2+-induced binding of protein kinase C in subcellular fractions is expressed in parentheses as the percentage of activity lost from the cytosol now recovered in that fraction.
° Detergent-extractable TPA-stabilized binding of protein kinase C in subcellular fractions is expressed in parentheses as the percentage of Ca2+-induced binding in that fraction.
amount of TPA-stabilized detergent-extractable protein kinase C binding was always much lower than Ca$^{2+}$-induced protein kinase C binding. Although the nuclear fraction showed a higher amount of Ca$^{2+}$-induced protein kinase C binding, TPA-stabilized binding was only 5–25% of this amount. However, in the plasma membrane mitochondria-enriched fraction, TPA-stabilized binding was as much as 25–90% of the Ca$^{2+}$-induced binding. Despite the microsomal fraction exhibiting a significant amount of Ca$^{2+}$-induced protein kinase C binding, no detectable amount of protein kinase C was bound to this fraction in a TPA-stabilized manner. Similar differences in Ca$^{2+}$-induced and TPA-stabilized protein kinase C binding were observed in binding studies using isolated subcellular fractions and purified protein kinase C. Further subfractionation of the mitochondrial-plasma membrane fraction from heart and kidney by isopycnic centrifugation using Percoll demonstrated that the mitochondrial fraction thus obtained exhibited only Ca$^{2+}$-induced protein kinase C binding but no detectable amount of TPA-stabilized binding. Similar observations have been made in PYS and B16 melanoma cells. Thus, TPA-stabilized binding of protein kinase C appears to be subcellular fraction specific. Ca$^{2+}$-induced reversible binding, on the other hand, is less specific, occurring in all the subcellular fractions studied. These findings are presented, however, not to establish the relative distribution of protein kinase C to specific subcellular organelles, but rather to further emphasize differences between Ca$^{2+}$-induced reversible binding and TPA-induced stable binding.

To better elucidate the nature of the protein kinase C receptor site(s) (domains) in the membrane, particulate fractions from PYS cells and rat heart were treated with trypsin and phospholipases, and then both Ca$^{2+}$- and TPA-stabilized bindings of protein kinase C to the enzyme-modified membranes were determined. Trypsin treatment of the particulate fraction did not affect the Ca$^{2+}$-induced reversible binding of protein kinase C in both PYS and heart (Table IV). However, phospholipase C treatment resulted in 73 and 100% decreases in Ca$^{2+}$-induced binding of protein kinase C in the PYS and heart particulate fractions, respectively. Phospholipase A$_2$ was slightly less effective in abolishing Ca$^{2+}$-induced binding, causing 54 and 9% decreases with treatment of PYS and heart particulate fractions, respectively. The effects of these treatments on TPA-stabilized binding were quite different. Trypsin treatment resulted in almost complete loss of TPA-stabilized binding with both PYS and heart particulate fractions. Phospholipase C treatment had little effect on TPA-mediated binding in the PYS particulate fraction but decreased protein kinase C binding to the heart particulate fraction by 76%. Phospholipase A$_2$ treatment caused a decrease in TPA-induced protein kinase C binding with both PYS (100%) and heart (77%) particulate preparations.

That there is little change in Ca$^{2+}$-induced binding of protein kinase C with trypsin treatment of membranes suggests that membrane proteins may be not involved in this type of protein kinase C binding. The decrease noted in this type of membrane binding after treatment with phospholipases does indicate that membrane phospholipids are involved in Ca$^{2+}$-induced binding. The lack of selectivity in Ca$^{2+}$-induced binding of protein kinase C to subcellular fractions coupled with reports showing Ca$^{2+}$-induced reversible binding of protein kinase C to PS-coupled affinity columns (33, 37), phenyl-Sepharose (38), and phenothiazine-coupled Sepharose suggests the possibility that Ca$^{2+}$-dependent association of protein kinase C to subcellular organelles may not occur solely to facilitate phosphorylation of subcellular organelle-associated proteins, but rather may also serve as a sequestering mechanism to remove protein kinase C from the cytosol to minimize (regulate) the phosphorylation of cytosolic proteins.

Modification of membrane composition by the above-mentioned enzymes was carried out to gain additional evidence for different protein kinase C binding membrane sites for Ca$^{2+}$- and TPA-stabilized association of protein kinase C with membranes and not to define the biochemical composition of the protein kinase C binding sites. The loss of TPA-stabilized membrane binding of protein kinase C with trypsin or phospholipase treatment of the isolated membranes does suggest that both membrane proteins and phospholipids are required at, or near, specific binding sites to mediate this type of membrane association. TPA-stabilized as well as diacylglycerol-mediated membrane association of protein kinase C likely represents a mechanism to facilitate the phosphorylation of membrane-associated proteins.

In other studies Ca$^{2+}$-induced reversible binding of protein kinase C was determined with PYS membranes previously saturated with TPA-stabilized protein kinase C. These pre-treated membranes inhibited less Ca$^{2+}$-induced protein kinase C binding by an amount equal to the amount of protein kinase C already bound in a TPA-stabilized manner. Since Ca$^{2+}$-induced reversible binding of protein kinase C is a rapid process compared to the slower and temperature-dependent TPA-stabilized membrane binding of protein kinase C, it is conceivable that when protein kinase C is added to the membranes in the presence of both Ca$^{2+}$ and TPA, it initially binds in a Ca$^{2+}$-induced reversible manner to membrane receptor sites. Subsequently, a fraction of this loosely associated reversibly bound protein kinase C, depending on the presence of other cofactors like specific membrane proteins and phospholipids, may be converted to a more stable form which remains bound even with the subsequent removal of Ca$^{2+}$, TPA, and lipid from the suspension buffer. Such favorable conditions may be present only in the plasma membrane and nuclear fractions but not in the mitochondrial and microsomal fractions. Hence the latter two subcellular fractions exhibit only reversible Ca$^{2+}$-induced protein kinase C binding.

The amount of endogenous Ca$^{2+}$-induced protein kinase C activity bound to the total particulate fraction prepared in the presence of Ca$^{2+}$ was found to be 30–50% lower than the amount of protein kinase C activity which could be bound

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**Table IV**

Ca$^{2+}$-induced and TPA-stabilized bindings of protein kinase C with enzymatically modified membranes

<table>
<thead>
<tr>
<th>Modification</th>
<th>PYS membranes</th>
<th>Heart membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca$^{2+}$</td>
<td>TPA st.</td>
</tr>
<tr>
<td></td>
<td>induced</td>
<td>stabilized</td>
</tr>
<tr>
<td>Control</td>
<td>1.45</td>
<td>0.79</td>
</tr>
<tr>
<td>Trypsin (50 μg/ml)</td>
<td>1.47</td>
<td>0.79</td>
</tr>
<tr>
<td>Phospholipase C 20</td>
<td>0.38</td>
<td>0.83</td>
</tr>
<tr>
<td>μg/ml</td>
<td>0.67</td>
<td>0.67</td>
</tr>
</tbody>
</table>

*Protein kinase C activity is expressed in units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H$_4$/min at 30 °C.
when the total particulate fraction was incubated with Ca\(^{2+}\) and 5-fold the amount of purified protein kinase C relative to the amount of endogenous enzyme found in the cells. However, the amount of TPA-stabilized protein kinase C binding to the total particulate fraction was about the same as the amount of TPA-stabilized protein kinase C binding observed when the particulate fraction was similarly incubated with TPA plus Ca\(^{2+}\) and 5-fold the endogenous amount of purified protein kinase C. This suggests the presence of limited specific high-affinity membrane binding sites whose saturation occurs at physiological amounts of protein kinase C with TPA treatment. In contrast, low-affinity Ca\(^{2+}\)-induced binding apparently is not saturable with physiological amounts of protein kinase C. The differences between Ca\(^{2+}\)-induced and TPA-stabilized membrane bindings of protein kinase C are summarized in Table V.

Possible Role of Endogenous Diacylglycerol in Mediating Membrane Association of Protein Kinase C

With crude homogenate preparations we were unable to elicit chelator-stable membrane association of protein kinase C with the addition of diolein. In experiments with purified protein kinase C and isolated membranes diolein was found to mediate stable protein kinase C binding although to a lesser extent than noted with TPA. Presumably, endogenous diacylglycerols also regulate the association of protein kinase C with membranes under certain physiological conditions. This is suggested by the recent observations that such agents as interleukin-2 (12), interleukin-3 (13), gonadotropin-releasing hormone (22), thyrotropin-releasing hormone (39), and the \(\alpha\)-adrenergic agonist phenylephrine (40), all of which are known to increase phosphatidylinositol turnover and thus elevate intracellular diacylglycerol, also cause an increase in membrane association of protein kinase C. However, this hormone-induced increase in protein kinase C membrane association is transient and less pronounced than the prolonged effect observed with TPA treatment.

Even though both diacylglycerol and TPA can serve to increase (activate) membrane associated protein kinase C, cells do not necessarily respond to these two effectors in an identical manner. For example, Kreutter et al. (41) recently reported that unlike TPA, 1-oleoyl 2-acetylgllycerol was unable to induce HL-60 leukemic cell maturation as measured by inhibition of cell growth, phagocytosis, adherence of cells to plastic, and loss of transferrin receptor activity. Although 1-oleoyl 2-acetylgllycerol does induce some effects similar to TPA in HL-60 cells, enhanced phosphorylation of 5 specific endogenous proteins and increased choline incorporation into lyso-phosphatidylcholine was noted only with TPA (41). The more transient nature of response of two new protein kinase C activation (membrane association) in response to changes in diacylglycerol as opposed to the more prolonged activation observed with TPA might account for at least some of the differences noted in response to treatment of cells with these modulators.

References

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15. TTPA-induced

Acknowledgments

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