Mechanism of Inhibition of Adenylate Cyclase by Phospholipase C-catalyzed Hydrolysis of Phosphatidylcholine

IN Volvement of A Pertussis Toxin-sensitive G Protein and Protein Kinase C

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The phospholipase C-mediated hydrolysis of phosphatidylcholine has been shown recently to be activated by a number of agonists. Muscarinic receptors, which trigger various signal transduction mechanisms including inhibition of adenylate cyclase through G, have been shown to be potent stimulants of this novel phospholipid degradative pathway. We demonstrate here, by exogenous addition of Bacillus cereus phosphatidylcholine-hydrolyzing phospholipase C, that phosphatidylcholine breakdown mimics the ability of carbachol to inhibit adenylate cyclase. This effect is sensitive to pertussis toxin and is entirely dependent on the presence of protein kinase C. This kinase is also required for the inhibition by carbachol of adenylate cyclase. These results suggest that the activation of phosphatidylcholine breakdown by phospholipase C may play an important role linking or favoring the coupling of muscarinic receptors to G. Results presented here also show that phospholipase C-mediated hydrolysis of phosphoinositides by exogenous addition of Bacillus thuringiensis phosphoinositide-hydrolyzing phospholipase C does not affect adenylate cyclase, despite the fact that protein kinase C is translocated to an extent similar to that produced by the hydrolysis of phosphatidylcholine. According to the results shown here, both phospholipases also differ in their ability to down-regulate protein kinase C as well as to phosphorylate p80 and to transmodulate the binding of epidermal growth factor receptor, two well established effects of protein kinase C in Swiss 3T3 fibroblasts. This emphasizes the complexity, from a functional point of view, of protein kinase C activation "in vivo."

The phospholipase C (PLC) -mediated degradation of phosphoinositides (PI) has been implicated in a number of cellular functions (for reviews, see Refs. 1 and 2). More recently, phosphodiesterase degradation of phosphatidylcholine (PC) has also been shown to be activated in response to a number of agonists (3) as well as oncogene products (4–6). Evidence supporting a role for this pathway in the control of cellular functionality has also been addressed recently (7).

We (8) and others (9, 10) have demonstrated that muscarinic activation triggers the phosphodiesterase-mediated degradation of PC, which potentially may be a signaling mechanism used by these agonists. The activation of muscarinic cholinergic receptors results in the triggering of various signal transduction pathways, including inhibition of adenylate cyclase (AC) through a G-protein sensitive to pertussis toxin (G) and activation of PI-specific PLC (11). Distinct muscarinic receptor isotypes have been shown to activate selectively both signaling mechanisms. Thus, whereas M1 and M3 receptors efficiently activate PI turnover and poorly regulate AC, M2 and M4 receptors display a completely opposite behavior (12, 13). Our results were consistent with a model whereby muscarinic agonists stimulated PLC-mediated PC hydrolysis through a G-protein insensitive to pertussis toxin (8). The activation of this novel phospholipid degradative pathway by muscarinic agonists took place without any detectable effect on PI turnover, but was concomitant with the inhibition of AC through G, (8). Therefore, it appears that muscarinic receptors coupled to PC-PLC activation are also able to inhibit AC. An attractive suggestion is that PC-PLC stimulation could be an intermediary step linking or favoring the coupling of muscarinic receptors to inhibition of AC. In the present study, we demonstrate that PLC-mediated breakdown of PC inhibits AC through G. Therefore, this report shows that PC-PLC action is sufficient to mimic the ability of a PC turnover activator, like muscarinic agonists, to promote a cellular effect (i.e., inhibition of AC). We also show here that this effect is completely dependent on PKC and that this kinase is also necessary for the inhibition of AC by CCho. These results strongly suggest that CCho activation of PLC-

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†‡‡‡ The abbreviations used are: PLC, phospholipase C; AC, adenylate cyclase; CCho, carbamylcholine; G, pertussis toxin-sensitive GTP-binding protein; IBMX, 3-isobutyl-1-methylxanthine; PC, phosphatidylcholine; PI, phosphoinositides; PKC, protein kinase C; PC-PLC, phosphatidylcholine-hydrolyzing phospholipase C; P-L-PLC, phosphoinositide-hydrolyzing phospholipase C; PM, phorbol myristate acetate; PBS, phosphate-buffered saline; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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mediated hydrolysis of PC could play an important role in the mechanism whereby muscarinic agonists inhibit AC.

**MATERIALS AND METHODS**

**Cell Cultures**—Swiss 3T3 fibroblasts (passage 123) were purchased from Flow Laboratories and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. SK-N-SH cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in the same medium. Cells were grown in standard tissue culture flasks in a humidified air/CO₂ (19:1) incubator at 37 °C. Cells were made quiescent by incubation for 24 h in serum-free medium supplemented with transferrin (5 µg/ml) and Na₂SO₃ (1 µM).

**Isolation of PC-PLC from Bacillus cereus**—*B. cereus* PC-PLC was purified from cultures of *B. cereus* SE-1 (14, 15) using DEAE-Sepharose Fast Flow and phenyl-Sepharose CL-4B and chromatography on PBE 94 columns. Polybuffers were removed by Sepharose CL-4B chromatography followed by dialysis. The enzyme preparation was concentrated by ultrafiltration on Diaflo YM5 membranes (Amicon Corp., Lexington, MA). The enzyme was purified to complete homogeneity as confirmed by SDS-polyacrylamide gel electrophoresis followed by silver staining. The specific activity of the purified enzyme was 1 U/µg (1 U = 1 µmol of 3-PG produced per minute).

**Cyclic AMP Assays**—Cells grown in 22-mm diameter plastic culture dishes (Costar, Cambridge, MA) were made quiescent by serum starvation as described above. After incubation for 10 min in the presence of 3-isobutyl-1-methylxanthine (1 mM), either cholera toxin (100 ng/ml), forskolin (10 µM), or isoproterenol (10 µM) were added, and the incubation was continued for another 10 min. Afterward, cells were treated with different stimuli for 3 min, and reactions were terminated by adding trichloacetic acid (10%, w/v). In some experiments, cells were treated with pertussis (400 ng/ml) or PMA (500 ng/ml) for 24 h. Cyclic AMP levels in thichloacetic acid-soluble extracts were determined using a commercially available kit (Du Pont-New England Nuclear, Boston, MA).

**Adenylate Cyclase Assay in Membrane Preparations**—Adenylate cyclase enzymatic activity was measured in Swiss 3T3 membrane preparations according to the general method of Salomon et al. (17). Briefly, quiescent Swiss 3T3 fibroblasts were detached from culture dishes and pelleted by centrifugation (600 × g, 10 min), after which cells were frozen and homogenized in homogenization buffer (50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 0.1% bovine serum albumin, 5 mM β-mercaptoethanol, and 2% LKB ampholytes). The enzyme was purified to complete homogeneity as confirmed by SDS-polyacrylamide gel electrophoresis (8% polyacrylamide) in the second dimension. Isotopic focusing gels contained 1.4% LKB ampholytes (pH 3.5–5) plus 0.6% LKB ampholytes (pH 3.5–10) to resolved highly acidic polypeptides. After electrophoresis, fixed gels were incubated with 125I-protein A (20) and washed with 0.2% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% polyacrylamide) in the second dimension. Isotopic focusing gels contained 1.4% LKB ampholytes (pH 3.5–5) plus 0.6% LKB ampholytes (pH 3.5–10) to resolved highly acidic polypeptides. After electrophoresis, fixed gels were dried, and radiolabeled proteins were visualized by autoradiography.

**Identification of Protein Kinase C by Immunoblotting**—Cell extracts containing 1 µg of total cell protein were obtained from Swiss 3T3 fibroblasts treated as described in the legend to Fig. 5. Following denaturation in SDS sample buffer, proteins were resolved in 10% SDS-polyacrylamide gels and then transferred electrophoretically onto a polyvinylidene difluoride membrane (Immobilon, Millipore Continental Water Systems, Bedford, MA). PKC was visualized as described previously by using [125I]-labeled protein A (20). Equal amounts of protein were loaded in the different lanes, and the verification that the transfer of protein from the different cell lines was quantitative was carried out by staining with Ponceau S prior to the visualization of PKC.

**Immunocytochemical Procedures**—Swiss 3T3 fibroblasts were seeded (10,000 cells/cm²) in glass coverslips coated with fibronectin (Calbiochem, La Jolla, CA), according to the experiments, grown in serum-containing medium, and 2 mM L-glutamine and 10% fetal bovine serum were supplemented. Afterward, different agents were added as described in the legend to Fig. 3. Coverslips were rinsed immediately in 3.7% formaldehyde, 0.1 M phosphate-buffered saline (PBS) for 5 min, after which they were rinsed in PBS several times and subsequently incubated in 0.1% Triton X-100 for 10 min. Afterward, coverslips were incubated with MC5 monoclonal anti-PKC antibody (1:10 dilution) for 60 min in a humid chamber. Afterward, coverslips were rinsed extensively in PBS, incubated with the second antibody, fluorescein-conjugated sheep anti-mouse IgG (Amersham International) (1:25 dilution) for 60 min, and rinsed extensively in PBS; then coverslips were mounted with glycero-containing 0.1% p-phenylenediamine (Sigma). All these procedures were carried out at room temperature. Purified mouse monoclonal antibody, clone MC5 (Amersham International), recognized an epitope within the overall primary structure of PKC that is contained within the amino acid sequence EGN-WK-EPFKK. The antibody recognized a 90 kDa protein, PKC, being the sole PKC isoform present in 3T3 fibroblasts (21).

**Estimation of Protein Kinase C Activity by Analysis of Endogenous 80-kDa Protein Phosphorylation**—Phosphorylation of endogenous proteins was performed as described (20). Briefly, confluent cultures in 60-mm dishes were rinsed with Tris-buffered saline (15 mM Tris-HCl, pH 7.4) and then incubated in phosphate-buffered saline (140 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 8.8 mM dextrose, 0.5% bovine serum albumin, 100 mM Hepes, pH 7.4) at 37 °C for 10 min and were stopped as described (17).

**Analysis of Products of Phosphodiesteratic Hydrolysis of PC**—Cells were isolated by homogenizing in buffer containing 50 mM Tris-HCl, 5 mM 3-mercaptoethanol, 1.5 mM EDTA, 0.05% bovine serum albumin, 5 mM β-mercaptoethanol, 2.5 µg/ml soybean trypsin inhibitor, 150 nM of cAMP ATPase (4) (2 × 10⁵ cpm), 5 mM MgCl₂, 20 mM creatine phosphate, 50 µg of creatine kinase, 5 mM theophylline, and 50-100 µg of membrane protein in a total volume of 200 µl. Other reagents were added according to the legend to Table II. Incubations were carried out at 37 °C for 10 min and were stopped as described (17).

**RESULTS AND DISCUSSION**

**Exogenous Phosphatidylcholine-hydrolyzing Phospholipase C Inhibits the Formation of cAMP in Swiss 3T3 Fibroblasts**—To examine the possible role of PLC-catalyzed hydrolysis of PC in the inhibition of AC by muscarinic agonists, we initially determined the effect of the exogenous addition of *B. cereus* PC-PLC on cAMP levels of cholera toxin-stimulated Swiss 3T3 fibroblasts. The assays were conducted in the presence of 1 mM IBMX, which inhibits phosphodiesterase activity, so
the changes observed in cAMP levels should largely reflect alterations in the rate of cAMP synthesis rather than its degradation. Results from Fig. 1A clearly indicate that addition of different doses of \( B. \) \textit{cereus} PC-PLC to fibroblast cultures dramatically inhibited the ability of cholera toxin to activate AC without any detectable effect on basal cAMP levels. The inhibition of AC by PC-PLC correlates with the PC-hydrolyzing ability of this enzyme (compare Fig. 1, A and B). A similar inhibitory effect of PC-PLC on AC was observed in cells treated with forskolin (10 \( \mu \)M) or isoproterenol (10 \( \mu \)M) (Fig. 2). Interestingly, the addition of \( B. \) \textit{cereus} PC-PLC to Swiss 3T3 fibroblasts promotes the hydrolysis of PC with little or no effect on other phospholipids (7). Therefore, PC hydrolysis by PLC conceivably can be considered responsible for the inhibition of AC in Swiss 3T3 fibroblasts.

**Involvement of G, in the Inhibition of Adenylate Cyclase by Phosphatidylcholine-hydrolyzing Phospholipase C**

Results from Fig. 2 indicate that pretreatment of Swiss 3T3 fibroblasts with pertussis toxin abolished the ability of CCho to inhibit cholera toxin-stimulated AC. This is in agreement with previously published results (8, 12, 13) and is consistent with the involvement of G, in this process. If PLC-mediated hydrolysis of PC is involved in the inhibition of AC by muscarinic agonists, its effect should be blocked by pertussis toxin. To address this possibility, cholera toxin-activated Swiss 3T3 fibroblasts, either untreated or treated with pertussis toxin, were stimulated with \( B. \) \textit{cereus} PC-PLC, and cAMP levels were determined thereafter. Results from Fig. 2 demonstrate that pretreatment of Swiss 3T3 fibroblasts with pertussis toxin blocked the inhibition of AC by exogenous \( B. \) \textit{cereus} PC-PLC. These results suggest that PC-PLC may be an intermediate step in G,-mediated inhibition of AC by CCho.

**Protein Kinase C Is Required for the Inhibition of Cholera Toxin-activated Adenylate Cyclase by \( B. \) \textit{cereus} PC-PLC and CCho**

Quiescent Swiss 3T3 fibroblasts either untreated or chronically exposed to PMA (500 ng/ml, 24 h) were pretreated with IBMX (1 mM, 10 min), after which cholera toxin (CT) (100 ng/ml) was added for 10 min. Afterward, either \( B. \) \textit{cereus} PC-PLC (0.5 unit/ml), PMA (100 ng/ml), or \( B. \) \textit{thuringiensis} PI-PLC (0.5 unit/ml) were added, and incubations were conducted for 3 min, after which reactions were stopped and cyclic AMP levels were determined as described under “Materials and Methods.” Neither CCho, PC-PLC, PMA, nor PI-PLC affected control (cells not treated with adenylate cyclase stimulants) cyclic AMP levels. Results are mean ± S.D. of three independent experiments with incubations in duplicate.

![Fig. 2. Effect of different treatments on cholera toxin-stimulated adenylate cyclase. Quiescent Swiss 3T3 fibroblasts, either untreated (empty bars) or treated for 24 h with pertussis toxin (100 ng/ml) (filled bars) were incubated with cholera toxin (C. Toxin) (100 ng/ml), forskolin (Fsk) (10 \( \mu \)M), or isoproterenol (10 \( \mu \)M), for 10 min after pretreatment with IBMX (1 mM, 10 min).](image)

**Table I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>cAMP pmol/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PMA</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>CT</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td>CT + CCho</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>CT + PC-PLC</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>CT + PMA</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>

Results are mean ± S.D. of three independent experiments with incubations in duplicate.
fibroblasts with PMA leads to the complete depletion of PKC (25). Results from Table I clearly show that *B. cereus* PC-PLC was unable to inhibit cholera toxin-activated AC in cells with down-regulated PKC levels. This indicates that PKC activation is a crucial event in the mechanism whereby PC hydrolysis inhibits AC in Swiss 3T3 fibroblasts. As a further proof of the involvement of PKC in this inhibitory pathway, cAMP levels were determined in cholera toxin-activated cells which had been treated acutely with PMA. Results from Fig. 2 clearly show that PMA inhibited cholera toxin-stimulated AC through a PKC-dependent mechanism with little or no effect on basal cAMP levels. Furthermore, pretreatment of Swiss 3T3 fibroblasts with pertussis toxin abolished the effect of PMA on cholera toxin-stimulated AC (Fig. 2). All these results together support the notion that PC hydrolysis inhibits AC through G, by a PKC-dependent mechanism.

If PC-PLC activation is an intermediary step in the inhibition of AC by CCho, the effect of CCho on such an inhibitory mechanism should be dependent on the presence of PKC. To investigate this possibility, we determined the effect of CCho on cholera toxin-activated fibroblasts that either had been untreated or exposed chronically to PMA. Results from Table I show that CCho is unable to inhibit AC in cells with down-regulated PKC levels. Therefore, these results demonstrate that PKC is required for the inhibition of AC by CCho, and they suggest that activation of PC-PLC by this agonist (8) is involved in the regulation of AC through G,. Whether or not the stimulation of PC-PLC is absolutely necessary for the inhibition of AC by muscarinic agonists must await the design of specific inhibitors of PC hydrolysis which would allow the evaluation of the functional repercussion of the blockade of this phospholipid degradative pathway.

**Table II**

<table>
<thead>
<tr>
<th>Effect of different treatments on the adenylate cyclase activity of Swiss 3T3 cell membranes</th>
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<tbody>
<tr>
<td>Membranes from quiescent Swiss 3T3 fibroblasts were prepared and adenylate cyclase activity was determined, as described under “Materials and Methods,” following treatment either with buffer control, CCho (20 mM), PI-PLC (0.5 unit/ml), or with PC-PLC (0.5 unit/ml). Incubations were carried out either in the absence (None) or in the presence of 10 μM isoproterenol plus 10 μM GTP (Iso). Results, expressed as picomoles of cAMP/min/mg of protein, are mean ± SD of three independent experiments with incubations in duplicate. Neither of the treatments affected control (without isoproterenol) levels of adenylate cyclase.</td>
</tr>
<tr>
<td>Cyclic AMP formed</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>pmol</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Iso</td>
</tr>
<tr>
<td>Iso + CCho</td>
</tr>
<tr>
<td>Iso + PI-PLC</td>
</tr>
<tr>
<td>Iso + PC-PLC</td>
</tr>
</tbody>
</table>

**Fig. 3. Immunofluorescence localization of protein kinase C in Swiss 3T3 fibroblasts treated with different agents.** Quiescent Swiss 3T3 fibroblasts were either untreated (A) or incubated for 3 min with either PMA (100 ng/ml) (B), *B. cereus* PC-PLC (0.5 unit/ml) (C), or *B. thuringiensis* PI-PLC (0.5 unit/ml) (D). Immunofluorescence signals were determined as described under “Materials and Methods.” Essentially identical results were obtained in three independent experiments. Bar, 1.7 μm.
no effect on either basal or cholera toxin-activated AC. These results are consistent with the notion that the negative regulation of AC by PC-PLC or PMA is specific to the action of these stimulants.

Exogenous Phosphatidylcholine-hydrolyzing Phospholipase C Inhibits Adenylate Cyclase in Washed Membrane Preparations—To assess inhibition of AC by PC-PLC directly, the conversion of [32P]ATP to [32P]cAMP was measured in membranes prepared from Swiss 3T3 fibroblasts. Results from Table II clearly show that CCho potently inhibits isoproterenol-stimulated AC in Swiss 3T3 fibroblast membranes, indicating the existence of a functional AC system in these membranes preparations. Interestingly, the addition of PC-PLC (0.5 unit/ml) for 10 min significantly inhibits isoproterenol-stimulated AC activity in these membrane preparations with no effect on basal AC activity (not shown). These results confirm that the effects of PC-PLC observed on cAMP levels of Swiss 3T3 fibroblasts stimulated by cholera toxin, forskolin, or isoproterenol can actually be accounted for by changes in AC. The addition of PI-PLC produced no effect on isoproterenol-activated AC (Table II), in accordance with the results obtained with cultured fibroblasts. Also, it is noteworthy that pretreatment of Swiss 3T3 fibroblasts for 24 h either with pertussis toxin (400 ng/ml) or PMA (500 ng/ml) prior to the preparation of membranes abolished the inhibition of AC by CCho and PC-PLC (data not shown).

Exogenous Addition of Either Phosphatidylcholine or Phosphoinositide-hydrolyzing Phospholipases Modulate Protein Kinase C—To substantiate that the lack of inhibitory effect of PI-PLC on AC was not due to a theoretical inability of this treatment to activate PKC, we measured the translocation of this kinase to the plasma membrane of Swiss 3T3 fibroblasts by immunocytochemistry using a monoclonal anti-PKC antibody. This technique allows the detection of the organellar distribution of PKC “in situ” (26). Results from Fig. 3 show that both PC-PLC (panel C) and PI-PLC (panel D) provoked the translocation of PKC to the plasma membrane to an extent similar to that produced by PMA (panel B). PC-PLC addition was also able to promote PKC translocation when these experiments were carried out in the absence of extracellular Ca2+ (data not shown), which rules out the possibility that PC-PLC-induced translocation of PKC could be ascribed...
cent Swiss 3T3 fibroblasts were either untreated with different agents on protein kinase 4). Visualized as described under "Materials and Methods." Essentially identical results were obtained in three independent experiments.

As discussed above, chronic treatment of cells with PMA leads to the complete depletion of PKC by proteolytic degradation. Thus, it was of interest to elucidate whether or not PI-PLC and PC-PLC were equally potent in causing PKC down-regulation. To this aim, we chronically treated Swiss 3T3 fibroblasts with either PMA, PC-PLC, or PI-PLC, and we examined PKC distribution by immunocytochemistry, either before or after an acute challenge with PMA. From Fig. 4, it is clear that under these conditions no immunofluorescence signal was detected on the plasma membrane of cells that had been treated chronically with PMA or PI-PLC, either before (panels C and G) or after (panels D and H) acute challenge with PMA. Interestingly, cells that had been treated chronically with PC-PLC displayed an increased basal PKC translocation to the plasma membrane (panel E) similar to that detected in cells acutely stimulated with PMA, PI-PLC, or PC-PLC. Furthermore, the acute addition of PMA to cells that had been exposed chronically to PC-PLC promoted a further increase in the immunofluorescence signal on the plasma membrane (panel F). These results demonstrate that PMA and PI-PLC promote PKC down-regulation, whereas PC-PLC does not. As an independent verification of the different ability of PC-PLC, on the one hand, and PI-PLC and PMA, on the other, to down-regulate PKC, the following experiment was carried out. Cell lysates were prepared from Swiss 3T3 fibroblasts that either have been untreated or chronically exposed to PMA (500 ng/ml), PI-PLC (0.5 unit/ml), or PC-PLC (0.5 unit/ml). Afterward, PKC protein levels were determined by immunoblotting. Results from Fig. 5 show that, whereas PKC protein levels were reduced severely in PMA- and PI-PLC-treated cultures, PC-PLC induced little or no effect on this parameter. This, together with the fact that PC-PLC and PMA inhibit AC whereas PI-PLC is unable to do so, and keeping in mind that all three stimulants were equally potent in inducing PKC translocation, suggests that the functional repercussion of PKC activation depends on the nature of the triggering agent.

It would be of interest to examine whether stimulation by either PLC would also differ in the ability to activate other well-established PKC substrates. To address this issue, we determined two additional parameters of PKC activity in this study, the functional repercussion of PKC activation depends on the nature of the triggering agent.

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| Table III |
| Effect of different treatments on $[^{125}]$I-EGF binding to Swiss 3T3 fibroblasts |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$[^{125}]$I-EGF bound (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$4150 \pm 230$</td>
</tr>
<tr>
<td>PMA</td>
<td>$1700 \pm 150$</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>$2100 \pm 160$</td>
</tr>
<tr>
<td>PC-PLC</td>
<td>$4300 \pm 320$</td>
</tr>
</tbody>
</table>

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induced a dramatic increase in p80 phosphorylation (compare panels A, B, and D). The ability of these two stimulants to induce the phosphorylation of p80 was abolished completely in cells with PKC down-regulated levels (panels E, F, and H). Interestingly, addition of PC-PLC did not induce any alteration in p80 phosphorylation (panels B and F). Another sensitive measure of PKC activation "in vivo" is the down-regulation of EGF receptors (28), which can be quantitated by a decrease in 125I-EGF binding. As shown in Table III, PMA and PI-PLC induced a dramatic decrease in EGF receptors whereas addition of PC-PLC showed no detectable changes in 125I-EGF binding.

All these results clearly support the notion that activation with PI-PLC or PC-PLC both promote PKC translocation but that the ultimate functional effect of such a response is different in both cases. Thus, whereas PC-PLC inhibits AC but is unable to induce p80 phosphorylation, to modulate EGF binding, or to induce PKC down-regulation, PI-PLC displays a completely opposite behavior. Interestingly PMA induces mutations which can be quantitated "in vivo," whereas addition of PC-PLC showed no detectable changes in lz5I-EGF binding. As shown in Table I1, PMA and PI-PLC induced a dramatic increase in p80 phosphorylation (compare panels A, B, and D). The ability of these two stimulants to induce the phosphorylation of p80 was abolished completely in cells with PKC down-regulated levels (panels E, F, and H). Interestingly, addition of PC-PLC did not induce any alteration in p80 phosphorylation (panels B and F). Another sensitive measure of PKC activation "in vivo" is the down-regulation of EGF receptors (28), which can be quantitated by a decrease in 125I-EGF binding. As shown in Table III, PMA and PI-PLC induced a dramatic decrease in EGF receptors whereas addition of PC-PLC showed no detectable changes in 125I-EGF binding.

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