Differential Regulation of Phosphoinositide and Phosphatidylcholine Hydrolysis by Protein Kinase C-β1 Overexpression

EFFECTS ON STIMULATION BY α-THROMBIN, GUANOSINE 5′-O-(THIOTRIPHOSPHATE), AND CALCIUM*

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Rat 6 fibroblasts that stably overexpress cDNA for the β1 isozyme of protein kinase C (PKCβ3 cells) were used to determine the effect of protein kinase C (PKC) overexpression on hormonal stimulation of phospholipid hydrolysis. In control Rat 6 cells, inositol trisphosphate (InsP3) were increased 9-fold in 15 s in response to 10 nM α-thrombin, compared with only a 2-fold increase in PKCβ3 cells. PKC overexpression also inhibited thrombin-stimulated production of 1,2-diacylglycerol, the other product of phosphatidylinositol 4,5-bisphosphate hydrolysis, by 73% at 15 s. In permeabilized cells, PKC overexpression greatly reduced guanosine triphosphate-stimulated InsP3 accumulation, but did not affect InsP3 stimulation by increased free calcium concentration. These data suggest that desensitization of thrombin-stimulated phosphoinositide-phospholipase C is enhanced by PKC-β1 overexpression and may involve modulation of G-protein/phospholipase C coupling.

In contrast, thrombin was 4.5-fold more effective in stimulation of phosphatidylcholine-phospholipase D activity in PKCβ3 cells than in control cells, as determined by phosphatidylethanolamine formation. In permeabilized cells, guanosine thiotriphosphate also stimulated phospholipase D activity more effectively in PKCβ3 cells than in control cells, suggesting that up-regulation of phospholipase D activity by PKC overexpression occurs distal to the thrombin receptor. These results suggest that PKC may act as a switch to up-regulate phosphatidylcholine-phospholipase D and down-regulate phosphoinositide-phospholipase C stimulations.

Many extracellular hormone and neurotransmitter receptors transduce signals into cells through activation of phosphatidylinositol-specific phospholipase C (PI-PLC)† (1). PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to yield two second messengers: inositol 1,4,5-trisphosphate, which mobilizes intracellular calcium (2), and 1,2-diacylglycerol (DAG), which activates protein kinase C (PKC) (3). More recently, it has been found that many receptors that are coupled to PI-PLC also stimulate phospholipase D (PC-PLD) activity, hydrolyzing phosphatidylcholine (PC) directly to phosphatidic acid (4), which may be subsequently converted to DAG (5).

Although the stimulation of protein kinase C by DAG has been well characterized (6), less is known about the feedback regulation of PI-PLC and PC-PLD activities by PKC. Previous studies of this feedback regulation have relied mainly on pharmacological approaches to assess PKC function, such as short term activation of PKC with phorbol esters, inhibition with PKC inhibitors (7-11), or down-regulation of PKC by chronic exposure of cells to phorbol esters (12, 13). However, phorbol esters have been reported to exert some effects that may be independent of PKC stimulation (14, 15), and it has been suggested that effects of phorbol esters on phosphoinositide and PC hydrolysis are not mediated by PKC (16, 17). In addition, these pharmacological approaches do not allow for investigation of the roles of individual PKC isozymes. We have previously used Rat 6 fibroblasts that stably overexpress the β1 isozyme of PKC (18) to study the feedback regulation of phosphor ester-stimulated phospholipase activity, and we found that PKC overexpression enhances phorbol ester-stimulated PC-PLD activity (19). We now report the effects of PKC-β1 overexpression on thrombin-, guanine nucleotide-, and calcium-stimulated PI-PLC and PC-PLD activities. Our results show that PKC overexpression down-regulates agonist-stimulated phosphoinositide-specific phospholipase C activity with concomitant up-regulation of agonist-stimulated PC-PLD activity. These effects appear to occur, at least in part, through regulation of the coupling of regulatory GTP-binding protein(s) (G-proteins) to catalytic activity.

EXPERIMENTAL PROCEDURES

Materials—Myo-[2-3H]inositol and [9,10-3H]myristic acid were from Amer sham Corp. [20-3H]Phorbol-12,13-dibutyrate (PDBu) was from Du Pont-New England Nuclear. AG1-X8 resin (formate form) was from Bio-Rad. Precoated silica 60 plates were from Merck. Cell culture reagents were from Gibco. α-Thrombin, GTPγS, and all other reagents were from Sigma.

Measurement of Inositol Triphosphate, Phosphatidylethanol, and Diacylglycerol Formation—Control (R6 C1) and PKC-β1-overexpressing cells (R6 PKC3) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum as previously described (19).

For measurement of [3H]inositol polyphosphate production, the
cells were labeled with 10 μCi/dish [3H]inositol in inositol-free DMEM with 0.5% serum for 48 h. For experiments with α-thrombin, labeled cells in 1 ml of serum-free DMEM received 20 μl of DMEM or concentrated drug. Reactions were terminated and inositol phosphates separated by anion exchange chromatography as previously described (10), except that InsP2 was eluted with 0.95 M ammonium formate, 0.1 M formic acid, rather than 1.0 M ammonium formate, 0.1 M formic acid to minimize contamination by InsP3. Data are expressed as percent of total chloroform-extractable [3H]inositol-labeled lipid (21).

To monitor phospholipase D activity, cellular PC pools were labeled for 2 h with [3H]myristate, and phosphatidylethanol (PEt) was separated by anion exchange chromatography as previously described (21). DAG mass levels of serum-deprived unlabeled cells were determined by conversion to [32P]phosphatidic acid using Escherichia coli DAG kinase as described previously (22) with minor modifications (11). DAG mass was normalized to phospholipid phosphate content of the extract (23).

**Cell Permeabilization**—For measurement of effects of GTPγS or free calcium concentration, cells were permeabilized with saponin as described previously (24) with slight modifications. After labeling with [3H]inositol or [3H]myristic acid as described above, cell monolayers were washed twice with serum-free DMEM, incubated at 37°C for 15 min, and then permeabilized for 5 min at 37°C with 20 μg/ml saponin in an intracellular buffer containing 110 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 3 mM Na2ATP, 8 mM creatine phosphate, 6 units/ml creatine kinase, 20 mM HEPES, pH 7.0, and 4 mM MgCl2, 1 mM EGTA, and 0.317 mM CaCl2, which were calculated to yield free calcium and magnesium concentrations of 0.1 mM and 1.25 mM, respectively. The dishes were then washed 3 times with intracellular buffer without saponin before treatment with drug in intracellular buffer as indicated. This permeabilization protocol rendered 86–89% of the extracts (23).

For experiments with varying free calcium concentrations, added CaCl2 and MgCl2 concentrations were calculated with a computer program to yield 1.25 mM free Mg2+ and the indicated [Ca2+]i in the presence of 1 mM EGTA and 3 mM ATP at pH 7.0.

**Phorbol Dibutyrate Binding**—To monitor the level of PKC expression during repeated passage of the cells, PKC was partially purified (through DEAE-Sephacel) from confluent cultures of R6 C1 and R6 PKC3 cells as described previously (18), and [3H]PDBu binding was measured using the procedure of Sando and Young (25).

### RESULTS

**Stimulation of Inositol Phosphates by Thrombin**—To assess the effects of protein kinase C overexpression on PI-PLC, thrombin-stimulated inositol phosphate levels were measured in control (R6 C1) and PKC-β1-overexpressing (R6 PKC3) cells. Fig. 1 shows time courses of the effects of 10 nM α-thrombin on [3H]InsP1, [3H]InsP2, [3H]InsP3, and [3H]InsP4 accumulation. α-Thrombin induced accumulation of all inositol phosphates measured in both cell types, but the stimulation was clearly diminished by PKC overexpression. Within 15 s, α-thrombin (10 nM) induced an 8.7-fold increase in the level of InsP3 in R6 C1 cells compared with only a 2.1-fold increase in R6 PKC3 cells (Fig. 2). Higher concentrations of thrombin were also less effective in R6 PKC3 cells than in R6 C1 cells at 15 s, although the InsP3 level in response to 100 nM thrombin still appeared to be rising in R6 PKC3 cells, while the response in control cells saturated at approximately 10 nmol thrombin. These data suggest a decrease in both potency and efficacy of thrombin in the R6 PKC3 cells.

**Stimulation of Phosphatidylethanol and Diacylglycerol Production by Thrombin**—Production of [3H]PET from [3H]myristate-labeled cells in the presence of 0.5% ethanol was measured as an index of phospholipase D-mediated hydrolysis of PC. PET has been shown to be produced exclusively through a phospholipase D-mediated transphosphatidylation reaction in which PC-PLD transfers the phosphatidyl group to ethanol rather than water (26). Fig. 3 shows time courses of α-thrombin-stimulated PET formation in R6 C1 and R6 PKC3 cells. α-Thrombin (9 nM) was clearly more effective in stimulating PET formation in R6 PKC3 cells (4.9-fold in 1 min) than in control cells (1.1-fold in 1 min).

The stimulation of 1,2-diacylglycerol mass levels, which may be produced through both phosphoinositide and PC...
metabolism (27), was also affected by PKC-β1 overexpression. The time course in Fig. 4 shows that 10 nM thrombin stimulated DAG production in a biphasic manner, as has been reported previously with fibroblasts (28). At early time points (15–30 s) that are thought to reflect stimulation of DAG production predominantly from phosphoinositide metabolism (27, 28), a rise in DAG mass was evident in both R6 C1 and R6 PKC3 cells. As with thrombin-stimulated InsP3 levels, this early DAG increase was clearly suppressed (73% inhibition) in R6 PKC3 cells relative to control cells. A second peak shown that 10 nM thrombin stimulation by PKC overexpression occurs at a level after the thrombin receptor, the effect of GTPγS on [3H]PEt formation was measured with permeabilized R6 C1 and R6 PKC3 cells. The dynamic range of stimulatory free calcium concentration (0.1–100 μM) is similar to that observed previously for phosphatidylinositol 4,5-bisphosphate hydrolysis by purified PI-PLC (29) or by membrane preparations (30).

To determine whether the up-regulation of PC-PLD activity by PKC overexpression occurs at a level after the thrombin receptor, the effect of GTPγS on [3H]PEt formation was measured with permeabilized R6 C1 and R6 PKC3 cells. GTPγS stimulated PEt formation in both cell types, with no effect on inositol polyphosphate levels (not shown). Fig. 5 shows that in saponin-permeabilized R6 C1 and R6 PKC3 cells, GTPγS over a range of 0.1–100 μM concentration stimulated InsP3 levels. As observed with thrombin-stimulated inositol phosphates, GTPγS was less efficacious in R6 PKC3 cells (5-fold stimulation by 100 μM GTPγS at 5 min) than in R6 C1 cells (27-fold stimulation by 100 μM GTPγS at 5 min) in the stimulation of InsP3 levels (data not shown).

Since Ca²⁺ has been shown to stimulate isolated PI-PLC directly (29), we measured the effect of PKC overexpression on free calcium-stimulated inositol phosphate production as an index of PI-PLC activity distal to regulation by agonist receptors and G-proteins. Increasing free calcium concentrations stimulated [3H]InsP3 accumulation (Fig. 6) to a similar extent in saponin-permeabilized R6 C1 and R6 PKC3 cells. The dynamic range of stimulatory free calcium concentration (0.1–100 μM) is similar to that observed previously for phosphatidylinositol 4,5-bisphosphate hydrolysis by purified PI-PLC (29) or by membrane preparations (30).

To determine whether the up-regulation of PC-PLD activity by PKC overexpression occurs at a level after the thrombin receptor, the effect of GTPγS on [3H]PEt formation was measured with permeabilized R6 C1 and R6 PKC3 cells. GTPγS stimulated PEt formation in both cell types, with maximal stimulation occurring at 30–100 μM GTPγS (Fig. 7). This maximally effective concentration of GTPγS is similar to that required for maximal stimulation of InsP3 accumulation. As observed with thrombin, GTPγS was more effective
Effects of PKC overexpression on GTPγS- or calcium-stimulated PI-PLC and PC-PLD were measured to identify the step(s) subject to regulation by PKC. Thrombin is thought to stimulate PI-PLC activity through interaction with a G-protein-coupled receptor (33, 34). The inhibition of thrombin- and GTPγS-stimulated, but not free calcium-stimulated, InsP3 formation by PKC overexpression suggests that the main inhibitory effect of PKC is on the G-protein or the coupling of the G-protein to PI-PLC. Because elevated free calcium concentration stimulates purified PI-PLC activity directly (29), the lack of inhibition of calcium-stimulated PI-PLC activity by PKC overexpression indicates that regulation by PKC occurs prior to the PI-PLC enzyme. Similar conclusions were reached by Orellana et al. (31) who reported that phorbol ester treatment of astrocytoma cells significantly inhibited GTPγS-stimulated, but not calcium-stimulated, formation of inositol phosphates. More recently, it was shown that phorbol ester treatment of several cell types stimulated phosphorylation of PI-PLC-β but not phosphorylation of PI-PLC-α or PI-PLC-γ (32). Interestingly, PI-PLC-β is the only PI-PLC isozyme that has been shown to be regulated by a heterotrimeric G-protein (35). The observation that in vitro phosphorylation of PI-PLC-β by PKC does not affect its activity has led Ryu et al. (32) to propose that this phosphorylation may prevent its activation by G-proteins.

Our observation that PKC overexpression enhances stimulation of PEt formation by α-thrombin and by GTPγS to similar extents suggests that stimulation of PC-PLD is also regulated by PKC at a level distal to the hormone receptor. This finding extends previous reports that phorbol ester treatment significantly enhanced PC-PLD activity in the presence of GTPγS but had little potentiating activity in the absence of GTPγS (9). Although PKC overexpression clearly enhanced α-thrombin-stimulated [3H]PEt formation (Fig. 3), a corresponding effect of PKC overexpression on [3H]phosphatidic acid production was not reproducibly observed in the presence or absence of ethanol (not shown). Presumably, [3H]PEt production is easily measurable because it is a stable end product (26), while phosphatidic acid, the physiological product of PC-PLD activity, is rapidly converted to metabolites such as CDP-diacylglycerol (36) and the putative secondary messenger lysophosphatidic acid (37, 38). In contrast to results in phorbol ester-stimulated fibroblasts (19), phosphatidic acid does not appear to be dephosphorylated to DAG to a measurable extent in thrombin-stimulated fibroblasts, since ethanol, which decreases phosphatidic acid formation by diverting phosphatidyl groups into PEt, has no effect on α-
thrombin-stimulated DAG mass levels. Thus, the second phase of DAG mass stimulated by α-thrombin is probably derived through pathways other than the sequential action of PC-PLD and phosphatidic acid phosphohydrolase. Consistent with this, the second phase of DAG mass is not enhanced by PKC overexpression (Fig. 4), despite the observed enhancement of PC-PLD activity (Fig. 3).

Scheme I shows a working model for the regulation of PI-PLC and PC-PLD activities by PKC. Stimulation of the thrombin receptor by α-thrombin activates PI-PLC via a regulatory G-protein, perhaps Gαi (35). PI-PLC hydrolyzesPIP2 to yield InsP3 and DAG, resulting in stimulation of PKC. PKC then feeds back to inhibit further G-protein coupling to PI-PLC and to enhance G-protein coupling to PC-PLD activity. This shift from PI-PLC activity during the initial phase of the cellular response to PC-PLD activity during the later phase may serve to preserve cellular PIP2, which is less abundant than PC (39, 40). Alternatively, InsP3 and DAG, the products of PI-PLC activity, may be necessary for initiation of cellular responsiveness to hormones such as α-thrombin, while phosphatidic acid and its metabolites, which are derived from PC-PLD activity, may be necessary for sustenance of cellular responses.

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