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Rapid Heterologous Desensitization of Muscarinic and Thrombin Receptor-Mediated Phospholipase D Activation

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SUMMARY

Activation of the M_3 muscarinic receptor in 1321N1 human astrocytoma cells leads to increased phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine, which is maximal within 1 min of exposure to agonist. Studies examining the kinetics of phosphatidylethanol formation indicate that there is no further PLD activation beyond this time. Thrombin, a mitogen for quiescent 1321N1 cells, also activates PLD only transiently. The PLD response does not recover for up to 1 hr and cells that have been exposed to carbachol or thrombin do not respond to subsequent challenge with the heterologous agonist. In contrast to the desensitization observed with agonists, phorbol-12-myristate-13-acetate induces a sustained stimulation of PLD. In addition, cells pretreated with carbachol or thrombin show a normal response to phorbol-12-myristate-13-acetate, suggesting that the enzymatic activity of PLD is not compromised. Guanosine-5′-*O*-(3-thio)triphosphate activation of PLD in cell-free homogenates is also unaffected by prior treatment of the cells with agonist. Agonist-stimulated PLD activation in 1321N1 cells is mediated by protein kinase C (PKC). Thrombin and carbachol cause comparable changes in redistribution of both PKC-α and PKC-ϵ. The increase in membrane-associated PKC-α is transient and is reinitiated by addition of the heterologous agonist, whereas PKC-ϵ remains membrane associated for at least 60 min and is not further increased by addition of the heterologous agonist. We suggest that desensitization of PLD activation results from the down-regulation of an as yet undefined mediator required for agonist receptor coupling to PLD and that PKC-ϵ might participate in this down-regulation.

A common pathway for signal transduction in mammalian cells utilizes second messengers generated by hydrolysis of membrane phospholipids. Thus far three phospholipases, namely phospholipase A_2, PLC, and PLD, have been shown to be stimulated after activation of specific receptors located on the cell surface (1–3). These phospholipases are often activated by the same agonists, although the mechanisms of activation are not identical.

Cleavage of polyphosphoinositide by receptor-mediated activation of PLC produces inositol-1,4,5-trisphosphate, which mobilizes calcium from intracellular stores, and DAG, which stimulates PKC (4). There is general agreement that the polyphosphoinositides are the main source of DAG formed immediately after exposure to agonist and that DAG is, in turn, responsible for the initial activation of PKC (5). Hydrolysis of PC may, however, provide an additional source of long-term elevated DAG levels through two distinct pathways, (a) via hydrolysis by a PC-specific PLC to yield phosphocholine and DAG and/or (b) via hydrolysis by a PC-specific PLD to yield choline and PA. Sustained levels of DAG may play important roles in later cellular responses (6, 7). Several groups have reported that, in addition to serving as a precursor of DAG, PA (or its derivative lyso-PA) elicits biological responses directly. Various roles have been proposed for PA, including inhibition of adenylate cyclase (8), inhibition of $p21^{ras}$ GTPase-activating protein (9), and activation of kinases in a manner analogous to that of DAG (10). Additionally, lyso-PA has been shown to activate Ras, stimulate mitogen-activated protein kinase activity, and initiate DNA synthesis in fibroblasts (11–13).

In view of the potential importance of PLD-stimulated hydrolysis of PC for production of second messengers such as DAG and PA, it is crucial to understand the mechanism(s) by which PLD is regulated. There is now abundant evidence for the involvement of PKC, as well as the participation of a G protein, in PLD activation (see Refs. 3 and 14 for reviews). However, precise definition of the biochemical changes leading to PLD activation is still lacking.

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ABBREVIATIONS: PLC, phospholipase C; PLD, phospholipase D; DAG, diacylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; DMEM, Dulbecco's modified Eagle's medium; PEt, phosphatidylethanol; ARF, ADP-ribosylation factor; PMA, phorbol-12-myristate-13-acetate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; EtOH, ethanol.

An unexpected phenomenon that has not been specifically characterized is that agonist-stimulated activation of PLD is rapidly desensitized. Understanding this process could provide insight into the pathways for PLD activation and into means of altering the duration of signaling via this pathway. In the present study, we demonstrate that stimulation of PLD by either mitogenic (thrombin) or nonmitogenic (carbachol) agonists is rapidly desensitized and that the receptor-mediated desensitization is heterologous with respect to agonist. We present evidence that desensitization of receptor-mediated PLD activation does not occur at the level of the receptor, PLD, or the G protein that couples to PLD. Additional experiments suggest that desensitization is not explained by a loss of receptor-stimulated phosphatidylinositol bisphosphate hydrolysis, Ca^{2+} mobilization, or PKC activation. We consider possible mechanisms that may underlie the heterologous desensitization of receptor-mediated PLD activity.

Experimental Procedures

Cell culture. 1321N1 Astrocytoma cells were plated on 35-mm or 100-mm plates at 1–2 × 10^4 cells/cm^2, in DMEM supplemented with 5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, and were grown for 3–5 days at 37° in 10% CO_2.

PLD activity in intact cells. Astrocytoma cells grown in 35-mm plates were labeled for 18 hr in 1 ml of serum-free DMEM containing 3 μCi of [^{3}H]myristic acid/plate. The cells were then incubated with the appropriate agonist or vehicle plus 1% EtOH at 37°. Reactions were terminated by replacement of the medium with 1 ml of ice-cold methanol. Cells were immediately harvested into glass tubes. Plates were washed with an additional 1 ml of methanol and the samples were combined. The lipids were extracted according to the method originally described by Bligh and Dyer (15), dried under N_2, and spotted onto silica gel 60 thin layer chromatography plates. Plates were developed in an organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10), which separated PEt (R_F 0.32) and PA (R_F 0.21) from other phospholipids and neutral lipids. [^{3}H]PEt and [^{3}H]PA were identified using nonlabeled standards, scraped off, and quantified by liquid scintillation counting. For time course experiments, agonist or vehicle was added to the cells at time 0 and EtOH was added to the cells at the times indicated. For the desensitization experiments, cells were pretreated with agonist for 15 min, washed twice with 1 ml of DMEM, and incubated with agonist or vehicle plus EtOH for an additional 5 min at 37°. Reactions were terminated as described above.

Phosphoinositide hydrolysis assay. Cells were labeled with 1 μCi/ml *myo*-[^{3}H]inositol in serum-free DMEM for 18–24 hr. Carbachol (500 μM) was added at time 0 and lithium chloride (10 mM) was added at the indicated time. Reactions were terminated 5, 10, 15, 20, or 30 min later by the addition of 2 ml of acidified methanol (methanol/0.1 N HCl, 1:1). Plates were left on ice for 30 min and supernatant was removed. Total [^{3}H]inositol (poly)phosphates were eluted by formate Dowex anion exchange chromatography with 1 M NH_4COOH/0.1 M HCOOH.

***In vitro* PLD assay.** Cells grown in 100-mm plates were labeled for 18 hr in 5 ml of serum-free DMEM containing 5 μCi/ml [^{3}H] myristic acid. Cells were washed, scraped from the plates, and suspended in 1 ml of phosphate-buffered saline. After centrifugation at 500 × *g* for 5 min, the cell pellet was resuspended in 500 μl of lysis buffer (25 mM HEPES, 100 mM KCl, 3 mM NaCl, 6 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptin, 1 mM dithiothreitol) and sonicated on ice for 3 × 15 sec with a Kontes Micro Ultrasonic cell disrupter fitted with a microtip. After sedimentation of unbroken cells at 400 × *g* for 10 min, the resulting supernatant was used for the PLD assay. The assay was carried out at 37° for 30 min using 200 μg of cell protein in a final volume of 200 μl of the aforementioned lysis buffer containing 1 mM ATP, 1 μM free calcium, and 100 μM GTPγS. Reactions were terminated by the addition of 3 ml of chloroform/methanol (1:2, v/v). The lipids were extracted and PEt was isolated and quantified as described above. Protein concentrations were determined as described by Bradford (16).

Cell fractionation for PKC immunoblotting. Cells were treated with thrombin or carbachol, rinsed with ice-cold phosphate-buffered saline, and lysed by two cycles of freeze-thawing in 50 mM β-glycerophosphate buffer, pH 7.4, containing 1 mM EGTA and 340 μM $CaCl_2$ (approximately 50 nM free calcium), 0.3 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. The lysates were centrifuged at 37,000 × *g* for 10 min at 4°, and the resulting supernatant was saved as the soluble fraction. The pellet was resuspended and incubated on ice for 30 min in the same buffer containing 0.3% Triton X-100 but no added calcium, and the extract was centrifuged at 37,000 × *g* for 30 min. The supernatant was used as the particulate fraction. Aliquots containing 60 μg of protein were added to SDS sample buffer (17) and resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide). Samples were electrophoretically transferred to an Immobilon membrane, and nonspecific binding sites were blocked by incubation of the membrane with 3% bovine serum albumin. PKC-ε was detected by blotting with an isozyme-specific polyclonal antibody (1/500; a gift from Berta Strulovici, Tularik Inc., South San Francisco, CA). PKC-α, the only Ca^{2+}-dependent PKC isoform in 1321N1 cells (18), was detected with a monoclonal anti-PKC antibody (1/100; Amersham), followed by ^{125}I-Protein A. Autoradiographs were scanned with an LKB Ultroscan XL densitometer to quantify PKC immunoreactivity.

Results

Stimulation of PLC by thrombin and carbachol. PLD has the unique ability to facilitate the transfer of a phosphatidyl group to EtOH rather than water (3, 19). The kinetics of PLD activation by thrombin and carbachol were examined in [^{3}H] myristic acid-labeled astrocytoma cells using transphosphatidylation assays performed as described previously (20) and in Experimental Procedures. When EtOH is added simultaneously with either carbachol or thrombin (no preincubation) the product of the transphosphatidylation reaction, PEt, accumulates rapidly, indicating that both agonists activate PLD (Fig. 1). The formation of PEt is maximal within 5 min and no additional PEt accumulates beyond this time (Fig. 1) (20). PEt is considered to be metabolically stable over short times (21–23). The failure to see accumulation of PEt beyond 5 min therefore indicates that no additional PEt is formed. This is consistent with our previous data showing that [^{3}H]choline and [^{3}H]PA levels plateau within minutes of agonist addition (20) and with other studies[3] showing that PA levels, as measured by mass analysis, also plateau.

We also used the transphosphatidylation reaction to determine whether PLD is active at longer times after agonist addition. Cells were incubated with agonist for 15 min, 2.5 hr, or 5.0 hr, at which time 1% EtOH was added to the medium and the incubation was extended for an additional 5 min (Fig. 1 and see Fig. 4). If PLD remains active, PEt should accumulate in the presence of added EtOH. The failure to generate PEt after addition of EtOH at these later times demonstrates that there is no agonist-stimulated PLD activity.

In contrast, when cells are incubated with 1 μM PMA the accumulation of PEt during the first 30 min is slower and progressive (Fig. 2). With the exception of the earliest incubation times, PMA is more effective than either thrombin or carbachol at eliciting PEt formation. PMA remains effective

[3] Martinson and Brown, unpublished observations.

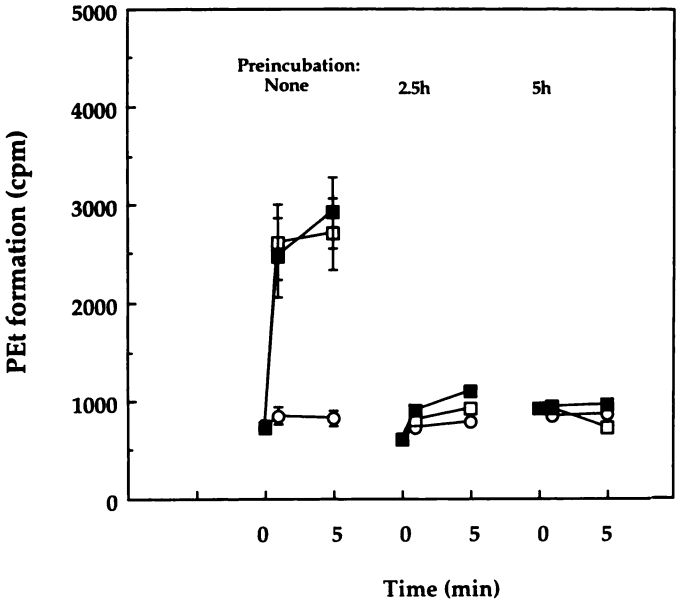

Fig. 1. Time course of [^{3}H]PEt formation in response to carbachol or thrombin. Cells were labeled overnight with [^{3}H]myristic acid and then exposed to vehicle (○), carbachol (■), or thrombin (□). EtOH (1%) was added to the medium either at the same time as carbachol or thrombin (no preincubation) or 2.5 or 5 hr after agonist addition. The incubation was stopped 1 or 5 min later and PEt was isolated and quantified as described in Experimental Procedures. Data are averaged values from one to three experiments, each performed in triplicate.

throughout the times studied and, although PLD enzymatic activity is somewhat reduced after 2–4.5 hr of preincubation with PMA, the rate of PEt accumulation is still markedly elevated over that of control cells (Fig. 2).

We also examined the ability of carbachol to stimulate phosphoinositide-specific PLC, as assessed by the accumulation of inositol monophosphate in the presence of LiCl. As shown in Fig. 3 and previously (24), there is continued inositol monophosphate accumulation over 30 min in the presence of agonist. This contrasts with the failure of agonist to increase PEt beyond the first 5 min of stimulation or at longer times. The maximal increase in inositol phosphate formation diminishes gradually from 5-fold over basal levels immediately after the addition of agonist to just 2-fold over basal levels after a 6-hr incubation period with carbachol, probably due to the development of receptor down-regulation (24).

Desensitization of PLD after thrombin or carbachol stimulation. Agonist-mediated desensitization may be classified as either homologous (only the response to the same agonist is attenuated) or heterologous (the response to any agonist is attenuated). Having established that thrombin and carbachol stimulate PLD activity with similar kinetics, we examined the relationship between the desensitization seen with these two agonists. Cells were pretreated with vehicle, carbachol, or thrombin for 15 min. After this treatment the medium was removed and replaced with fresh medium, and the cells were exposed to the same agonist or the heterologous agonist in the presence of 1% EtOH for 5 min. The data shown in Fig. 4 demonstrate that pretreatment with carbachol not only makes the cells refractory to the same agonist but also significantly reduces the response to thrombin. Carbachol pretreatment reduces the stimulation of PLD by thrombin by ~75%, and conversely thrombin pretreatment reduces the responses to carbachol by ~67%. Thus, receptor-mediated desensitization of PLD stimulation is heterologous. This observation indicates that desensitization does not occur primarily at the level of the specific receptor, although the greater reduction in the response to the homologous agonist probably reflects an added component of receptor desensitization.

In contrast to the effect of agonist pretreatment on subsequent agonist responses, pretreatment of the cells with agonist does not modify the ability of PMA to activate PLD (Fig. 5). Thus, PLD, when stimulated, can still catalyze the hydrolysis of its substrate.

Recovery of PLD activity after carbachol treatment. As shown in Fig. 4, treatment of the cells with carbachol or

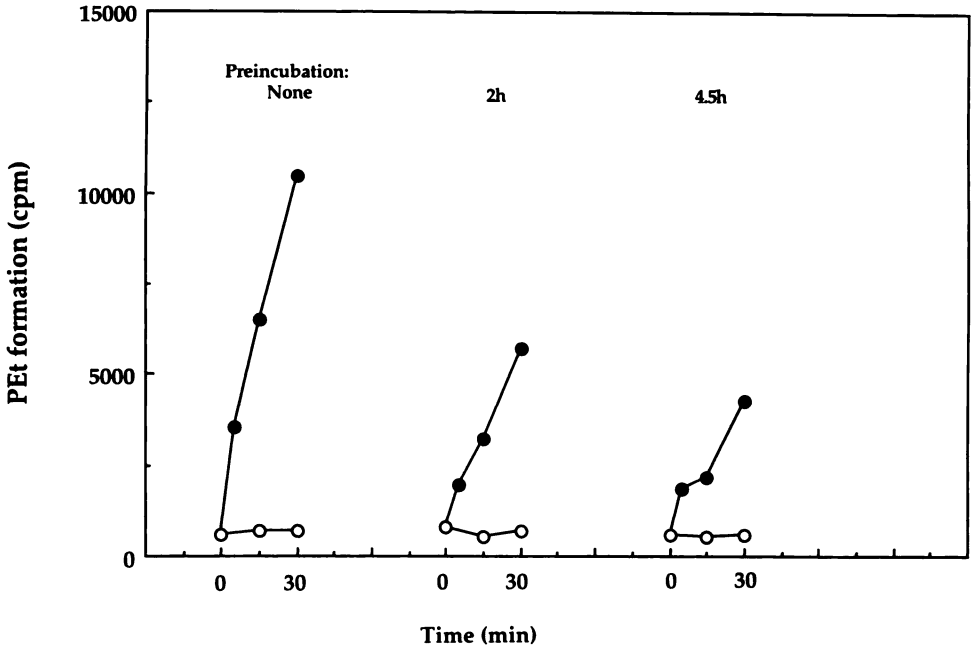

Fig. 2. Time course of [^{3}H]PEt formation in response to PMA. Cells were labeled overnight with [^{3}H]myristic acid and then exposed to 1 μM PMA (●) or dimethylsulfoxide (○). EtOH was added along with PMA (no preincubation) or 2 or 4.5 hr later. The incubation was stopped 5, 15, or 30 min after EtOH addition, and PEt was isolated and quantified. Data are averaged values from one of three experiments, each performed in triplicate.

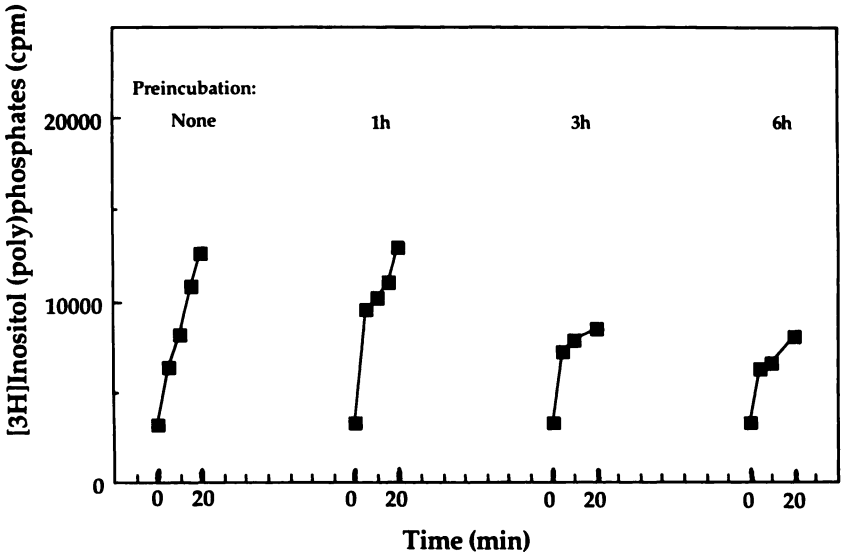

Fig. 3. Time course of phosphoinositide hydrolysis in response to carbachol. Cells were labeled overnight with *myo*-[^{3}H]inositol and then exposed to 500 μM carbachol. LiCl (10 mM) was added either along with carbachol (no preincubation) or after 1, 3, or 6 hr of carbachol treatment. The incubation was stopped 2–20 min later. Inositol phosphate formation was quantified as described in Experimental Procedures. Data are averaged values from one of three experiments, each performed in triplicate.

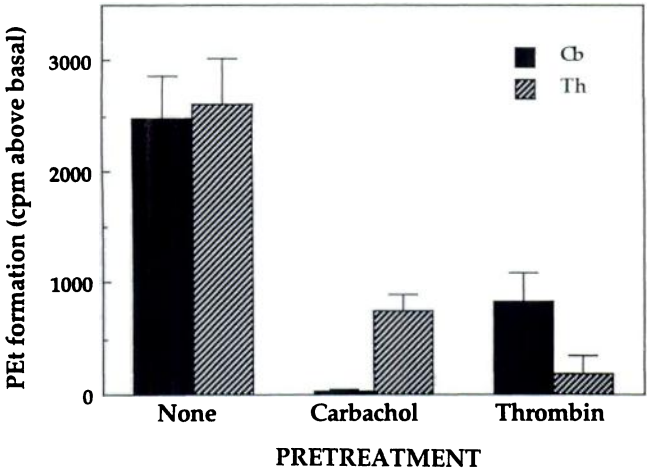

Fig. 4. Receptor-mediated desensitization of PLD activity. Cells were labeled with [^{3}H]myristic acid and pretreated with 500 μM carbachol (*Cb*), 0.5 unit/ml thrombin (*Th*), or vehicle (*None*) for 15 min. Cells were then washed and rechallenged for 5 min with the homologous or heterologous agonist in the presence of 1% EtOH, and PEt was isolated and quantified. Data are averages ± standard errors from two to five experiments, each performed in triplicate.

thrombin prevents PLD activation in response to an immediate rechallenge with another agonist. Previously we reported similar heterologous desensitization of Ca^{2+} mobilization in response to carbachol and histamine and demonstrated that it resulted from depletion of hormone-sensitive Ca^{2+} stores (25). Recovery of the Ca^{2+} response occurred within 30 min, provided that the agonist was removed and calcium was supplied in the medium. To determine whether the PLD response recovered under similar conditions, cells that had been incubated with carbachol for 15 min were washed several times to remove agonist and were allowed to recover for increasing times at 37° before being rechallenged with agonist and EtOH. As demonstrated in Fig. 6 and above, PEt is not formed when EtOH is added 15 min after addition of carbachol. Even when carbachol is washed out and cells are allowed to recover for up to 60 min in the presence of calcium, it is not possible to stimulate PEt formation (Fig. 6). It is therefore unlikely that failure to mobilize Ca^{2+} underlies the observed desensitization.

Putative G protein involvement in desensitization. Having established that carbachol- or thrombin-mediated desensitization of PLD appears to be independent of receptor down-regulation, Ca^{2+} mobilization, and phosphoinositide hydrolysis, we investigated the possibility that desensitization occurs at the level of a regulatory G protein. GTPγS has a stimulatory effect on PLD activity measured in cytosol-enriched membrane fractions (data not shown) or in cell homogenates (Fig. 7). Although we could not show potentiation by agonist, we asked whether the PEt response to GTPγS is down-regulated by either thrombin or muscarinic agonist pretreatment. The ability of 100 μM GTPγS to elicit PEt production was measured in sonicates prepared from control cells or cells that had been pretreated with either thrombin or carbachol. The extent of stimulation, albeit modest, is the same in control cells and in cells that were pretreated with thrombin or carbachol (Fig. 7). This suggests that agonist-mediated desensitization is not associated with a perturbation in G protein coupling to PLD.

PKC involvement in desensitization. We previously demonstrated that PKC is required for activation of PLD by carbachol in 1321N1 cells (20). To determine whether desensitization results from cells becoming refractory to agonist-induced PKC activation, we examined the redistribution of PKC-α and PKC-ε, the two major isoforms detected in 1321N1 cells, before and after agonist treatment. Previous studies from our laboratory demonstrated a rapid and transient redistribution of PKC-α in response to muscarinic receptor stimulation (18). Thrombin likewise induces a rapid increase in membrane-associated PKC-α, with kinetics of translocation similar to those seen for carbachol (Fig. 8).[4] Quantitative analysis of immunoblots obtained using antibodies that detect PKC-α demonstrates that maximal association of PKC-α with cell membranes occurs between 0.5 and 2 min after either thrombin (Fig. 8) or muscarinic receptor stimulation (18). The cellular distribution of immunoreactive PKC returns to that of control cells within 5 min. When cells that have been treated with thrombin for 15 min are washed and rechallenged with carbachol, carbachol is able to stimulate a secondary increase in

[4] Post *et al.*, manuscript in preparation.

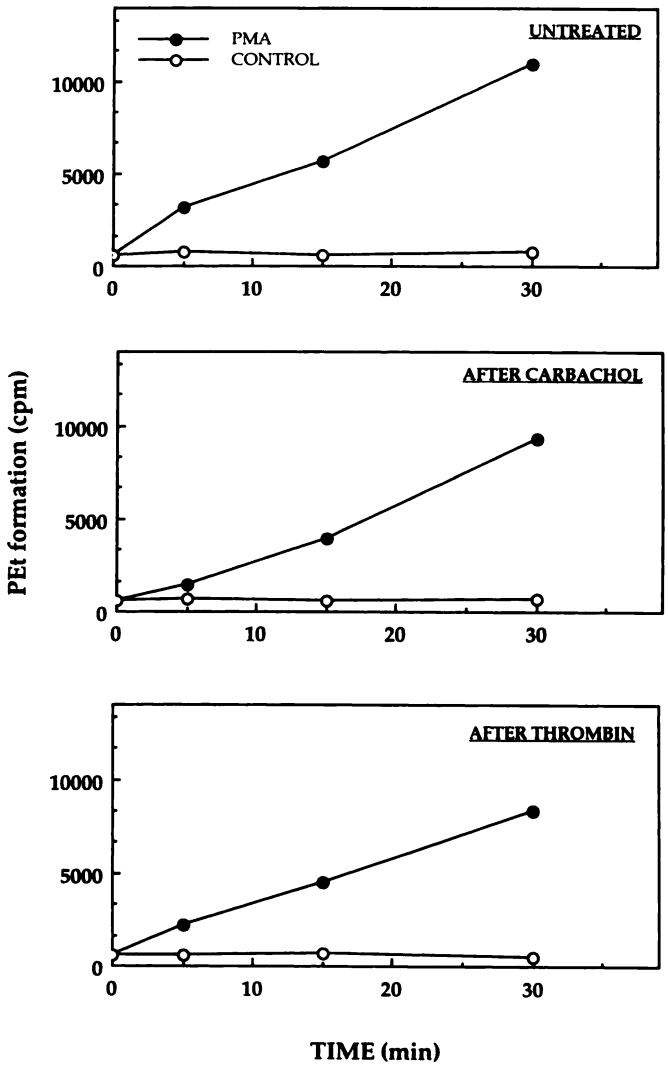

Fig. 5. Effect of carbachol and thrombin pretreatment on PLD activation by PMA. Cells were labeled overnight with [^{3}H]myristic acid and treated with 500 μM carbachol, 0.5 unit/ml thrombin, or vehicle (*UNTREATED*) for 15 min. The cells were then washed and exposed to 1 μM PMA (●) or dimethylsulfoxide (○) in the presence of 1% EtOH. At the indicated time (5, 15, or 30 min later) the incubation was stopped and the [^{3}H]PEt formed was isolated and quantified. Data are averaged values from one of two experiments, each performed in triplicate.

membrane-associated PKC-α (Fig. 8). Thus, desensitization of the PLD response is apparently not related to failure of the agonist to activate PKC-α. The kinetics of redistribution of PKC-ϵ, the major Ca^{2+}-independent isoform detected in 1321N1 cells, differ substantially from those observed for the α isoform. Agonist-induced membrane association of PKC-ϵ also increases rapidly but is sustained for at least 30 min, rather than being transient (Fig. 8).[4] Addition of the heterologous agonist (carbachol) 15 min after addition of thrombin does not further increase the already elevated membrane-associated PKC-ϵ immunoreactivity.

Discussion

Stimulation of either thrombin or muscarinic receptors activates PLD in 1321N1 astrocytoma cells. Using the accumulation of PEt, a transphosphatidylation product of PLD, as an indicator of PLD activity (3, 19, 21), we demonstrate that these agonists activate PLD with similar kinetics, indicative of rapid heterologous desensitization. The experiments presented here assess the potential involvement of several key mediators of the signal transduction pathway in this desensitization process.

First we considered the hypothesis that desensitization or down-regulation occurs at the receptor level. Several observations argue against this possibility. One is that coupling of the muscarinic receptor to phosphoinositide hydrolysis is sustained for at least the first hour after addition of carbachol, although muscarinic receptor-stimulated PEt formation is lost after 5 min. Inactivation of a subset of muscarinic receptors is unlikely, because 1321N1 cells contain almost exclusively receptors of the M_3 subtype (26) and we see no difference in the pharmacological characteristics of receptors mediating phosphoinositide hydrolysis and PLD activation.[5] In addition, the finding that stimulation of PLD by either thrombin or carbachol leads to heterologous desensitization to the other agonist argues that receptor down-regulation cannot be the primary mechanism by which PEt formation is turned off. Desensitization at the level of the receptor probably contributes to the decrease in the maximal inositol phosphate formation observed at longer times (Fig. 2) and the somewhat greater extent of homologous versus heterologous desensitization of PLD (Fig. 3). However, this appears to contribute little to the rapid heterologous loss of PEt formation.

G protein involvement in agonist-induced PLD activation has been demonstrated in neutrophils (27, 28), hepatocytes (19, 29), and HL-60 cells (30). Nonhydrolyzable guanine nucleotide analogs like GTPγS have been used extensively in the study of G protein involvement and have been shown to activate PLD in rat hepatocyte membranes (29, 31), in permeabilized endothelial cells (32), and in granulocyte homogenates (30), independently of phosphoinositide-specific PLC and PKC activation (see also Ref. 33). Recently two low molecular weight G proteins, ARF and a rho-like protein, have been suggested to be involved in PLD regulation (34–36). Our experiments demonstrate that PLD activation by GTPγS persists under conditions in which the cells have become refractory to further agonist-induced stimulation of PLD activity. This suggests that the heterotrimeric or low molecular weight G proteins are not uncoupled from PLD when the response is desensitized.

Although Ca^{2+} mobilization does not appear to be the major signal for PLD activation in 1321N1 cells (37), we tested the possibility that desensitization resulted from agonist-induced depletion of Ca^{2+} stores. In previous experiments monitoring intracellular calcium mobilization, we determined that cells respond fully to a second agonist challenge if they are first allowed to refill their depleted calcium stores (25, 38). However, when the same conditions were tested here, PLD activity remained refractory to further stimulation by carbachol. This observation indicates that the desensitization is not a consequence of diminished ability to mobilize Ca^{2+}.

The possibility that desensitization results from substrate depletion also appears unlikely, because we do not observe significant attenuation of PEt formation in cells chronically stimulated with PMA. Likewise, PLD itself does not appear to be desensitized, because its activity is not decreased in cells pretreated with either thrombin or carbachol and then stimulated with PMA. We cannot rule out the possibility that PMA

[5] Martinson and Brown, unpublished observations.

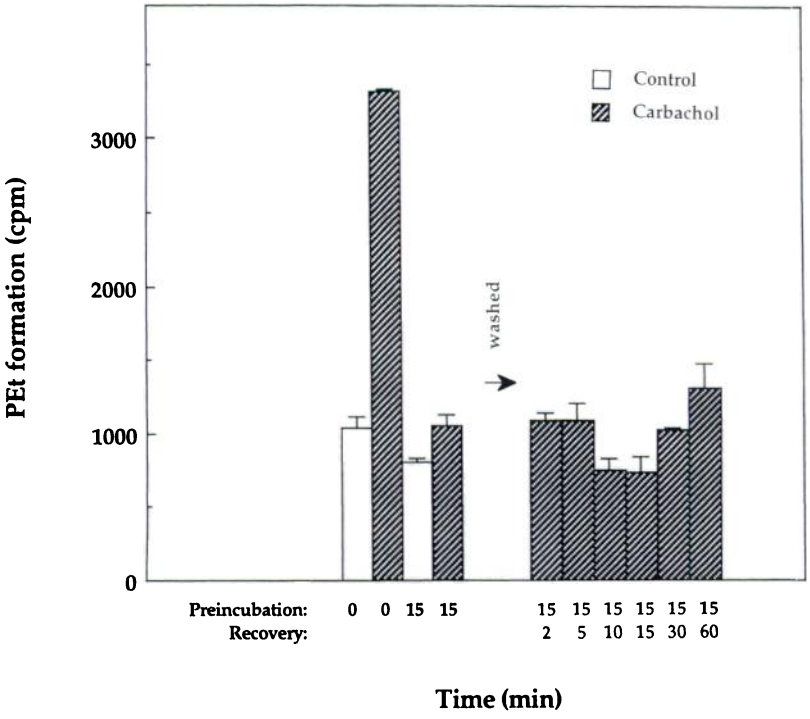

Fig. 6. Recovery of agonist-mediated PLD stimulation in 1321N1 astrocytoma cells. Cells that had been labeled with [^{3}H]myristic acid were incubated with 500 μM carbachol for 15 min. The cells were then washed several times to remove the agonist and were allowed to recover for 2–60 min at 37° before being rechallenged for 5 min with carbachol in the presence of 1% EtOH. Data are averages of triplicate samples from a representative experiment.

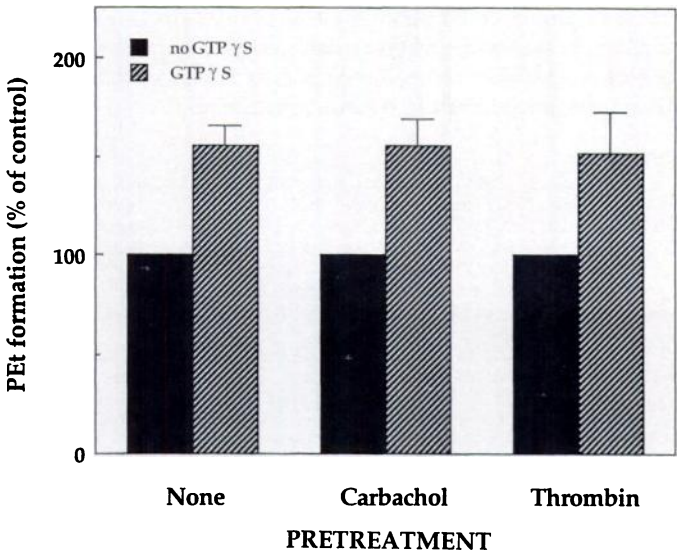

Fig. 7. Effect of agonist pretreatment on GTPγS-stimulated PLD activity in a cell-free system. Cells that had been labeled with [^{3}H]myristic acid were stimulated with 500 μM carbachol, 0.5 unit/ml thrombin, or vehicle (*None*) for 15 min. The medium was then removed, and the cells were scraped off the plate and sonicated. This homogenate was incubated with or without 100 μM GTPγS in the presence of 1% EtOH for 30 min at 37°. The reaction was stopped and the [^{3}H]PEt formed was isolated and quantified. Data are averages ± standard errors from four experiments, each performed in triplicate.

and G protein-linked receptors utilize different pools of PLD and different pools of substrate. However, data from recent studies with hepatocytes provide evidence that PMA-activated PLD and agonist-activated PLD utilize the same phospholipid substrate pool (23). In addition, as discussed below, the agonist-mediated PLD response is effected through activation of PKC and thus should be mimicked by PMA.

We have previously shown that PKC down-regulation inhibits the ability of carbachol to activate PLD, as measured by choline release, PEt formation, or DAG generation (20, 37). Although some reports suggest that receptor-mediated stimulation of PLD activity can occur independently of PKC activity (39, 40), PKC appears to be an obligate step in the stimulation of PLD in 1321N1 cells, as in other cell lines (41, 42). We therefore considered the possibility that PKC activation might be inhibited by treatment with the first agonist, providing a mechanism for heterologous desensitization. We examined agonist-induced redistribution of PKC, believed to be a prerequisite for enzyme activation, by immunoblotting, because this technique allows independent analysis of specific PKC isoforms. For PKC-α we found that, after the initial activation by thrombin or carbachol, a second transient increase in membrane-associated PKC was evoked by addition of the heterologous agonist. Thus, one would expect to see further activation of PLD under these conditions. For PKC-ϵ the effects of carbachol and thrombin on membrane-associated PKC were sustained. Addition of the heterologous agonist did not further increase membrane-associated PKC-ϵ, but because this isoform remained associated with the membrane it could be maintained in an activated state. Thus, desensitization is unlikely to result from failure of the agonist to activate PKC.

We also considered the possible role of protein tyrosine phosphorylation in the coupling of the receptor to PLD. Tyrosine kinase activity has been implicated in the activation of PLD in several systems (43), including neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine or granulocyte-macrophage colony-stimulating factor (44, 45), fibroblasts stimulated by epidermal growth factor or platelet-derived growth factor (46, 47), and HL-60 cells treated with peroxides of vanadate (48). Tyrosine kinase activity is also essential for thrombin-induced mitogenesis in 1321N1 cells (49). In studies testing three tyrosine kinase inhibitors (lavendustin, herbimycin A, and genistein), we did not observe any inhibition of

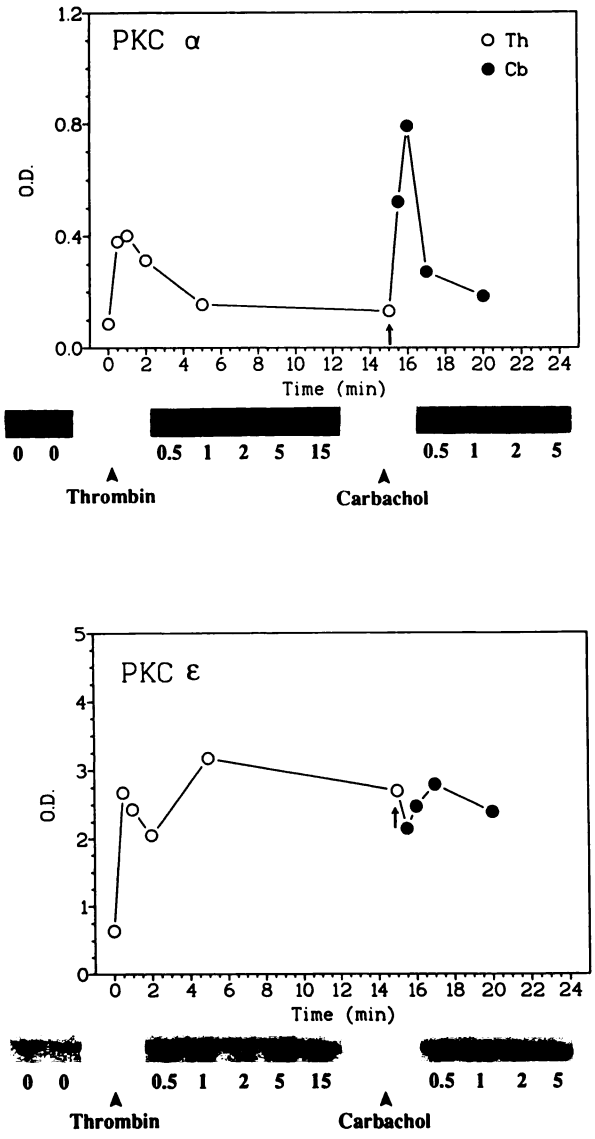

Fig. 8. Agonist-induced changes in membrane-associated PKC-α and PKC-ε immunoreactivity. Cells were treated with 0.5 unit/ml thrombin (*Th*) and harvested 0.5–15 min later (○) or treated with thrombin for 15 min, w ashed, exposed to carbachol (Cb), and harvested 0.5–5 min later (●). Membrane and cytosolic fractions were prepared and subjected to SDS gel electrophoresis, and PKC-α or PKC-ε immunoreactivity was determined as described in "Experimental Procedures." A representative gel showing changes in membrane-associated PKC is shown below the graphs. Arrows indicate the agonist that was added, and the number below each lane is the time (after agonist) at which the samples were harvested.

agonist-stimulated PLD activity (data not shown). It appears, on the basis of these results, that tyrosine phosphorylation is not a signaling mechanism involved in the activation of PLD in 1321N1 cells.

A working model for the activation of PLD activity should incorporate two well documented regulatory mechanisms. One is that the agonist stimulates PLD activity directly through a G protein; the other is that PLD is activated by PKC, which has been previously activated by the generation of phosphoinositide-derived DAG. The latter pathway has been proposed to regulate PLD activity in fibroblasts (50), endothelial cells (51), and other cells. We demonstrate here that PKC can still be activated (PKC-α) or remains activated (PKC-ε) when the PLD response to agonist is desensitized and that PMA can still regulate PLD at this time. Thus, it is most likely that desensitization occurs at the level of receptor regulation of a G protein. However, just as PKC- and G protein-regulated pathways may converge to control the activation of PLD (see Ref. 14), PKC may also contribute to the inactivation or desensitization of PLD. We previously demonstrated that PKC inhibits G protein (G_q)-mediated coupling of the muscarinic receptor to PLC and Ca^{2+} mobilization (52, 53) in 1321N1 cells. Similarly, although PKC may be involved in the initial stimulation of PLD, sustained PKC activation, as seen for the PKC-ε isoform, may inhibit continued receptor-mediated G protein-dependent activation of PLD.

The similar responses of 1321N1 astrocytoma cells to thrombin, a potent mitogen, and to carbachol, which is nonmitogenic in these cells, indicate that the two stimuli share the same lipid signaling pathways. This is supported by the observation that thrombin and carbachol do not have additive effects on PEt formation (data not shown). Our data also indicate that neither the increase in PC hydrolysis by PLD nor the second messengers that accumulate as a consequence of the activation of PLD are likely to account for the mitogenic capability of thrombin. A similar conclusion was reached by McKenzie *et al.* (54) for CCL39 fibroblasts. We are currently examining the involvement of other signaling components that may be recruited after activation of the thrombin receptor and are required to initiate DNA synthesis. In addition, the role of PKC-ε is being studied further, to determine whether its relatively sustained redistribution in response to agonist is part of the process by which receptor-stimulated PLD activity desensitizes.

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