Stimulation of Phosphatidylcholine Breakdown by Thrombin and Carbachol but Not by Tyrosine Kinase Receptor Ligands in Cells Transfected with M1 Muscarinic Receptors

RAPID DESENSITIZATION OF PHOSPHOCHOLINE-SPECIFIC (PC) PHOSPHOLIPASE D BUT SUSTAINED ACTIVITY OF PC-PHOSPHOLIPASE C

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In order to evaluate the possible contribution of phospholipase D (PLD) stimulation to the mitogenic response, a screening of a variety of different compounds (9-some of which are known to be potent mitogens, was performed using the well characterized Chinese hamster lung fibroblast (CCL39) cell line. In wild type CCL39 cells, or derivatives expressing high levels of either the human M1 muscarinic receptor (Hm1) or the human epidermal growth factor (EGF) receptor (39M1-81 and 39ER22 clones, respectively), thrombin, a potent mitogen for all three cell types, elicited the rapid activation of PLD (t1/2 activation, 30 s). Carbachol-mediated activation of the Hm1 receptor in the 39M1-81 clone, which is not a mitogenic signal, produced a similarly rapid although greater activation of PLD. Addition of EGF to the 39ER22 clone was able to provoke both a mitogenic response and stimulate PLD, albeit a comparatively small effect. In each case, the ability of PLD correlated with the stimulation of inositol phospholipid breakdown and was entirely dependent on the activation of protein kinase C. Moreover, the ability of both thrombin and carbachol to stimulate PLD was found to be rapidly desensitized, with a similar time course of desensitization (t1/2 desensitization, 90 s).

It has recently been reported that an increase in phospholipase C (PLC)-mediated phosphocholine (PC) hydrolysis by either addition of agonist or by extracellular addition of PC-specific PLC enzyme constitutes a mitogenic signal. In this regard, in addition to stimulation of PLD, thrombin and carbachol were both able to stimulate the activity of a phosphocholine-specific phospholipase C (PC-PLC), which did not appear to desensitize within the time course employed. By contrast, EGF was unable to elicit the stimulation of PC-PLC. Ligands such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), which bind to and activate receptors with intrinsic tyrosine kinase activity, are potent mitogens for CCL39 cells but were unable to stimulate either PLD or PC-PLC activity. Furthermore, exogenous addition of purified PC-PLC enzyme, although able to induce a strong and lasting hydrolysis of PC, was unable to produce a mitogenic signal on its own. On the basis of these results, we conclude that the activation of both PLD and PC-PLC is neither sufficient nor required to produce a mitogenic response.

Cells which have been rendered quiescent can be stimulated to re-enter the cell cycle by the addition of a variety of hormones or growth factors. This is not an "all or none" response, since several growth factors may mediate this effect on their own, whereas others can only synergize with pre-existing growth factors. The majority of hormones and neurotransmitters mediate their effect through the activation of a limited number of signal transduction systems which appear to be localized to the plasma membrane. One of the most widespread signal transduction systems under hormonal regulation involves the breakdown of inositol-containing phospholipids by a phosphatidylinositol-specific phospholipase C (PI-PLC) (1). The immediate products of this reaction are inositol 1,4,5 trisphosphate, which in concert with one of its intermediate breakdown products, inositol 1,3,4,5-tetakisphosphate, is capable of releasing Ca²⁺ from intracellular stores, and diacylglycerol (DAG), which together with Ca²⁺ promotes activation of protein kinase C (PKC) (1-3). PKC has been shown by several groups to play a role in mitogenic signaling (4-6), which highlights the role of DAG as being an important intermediate in cellular control. In addition to phosphoinositide breakdown, DAG can be produced by the activation of a more recently characterized phospholipase D (PLD) activity on phosphatidylcholine (PC) (7-9). The products of this reaction being choline and phosphatic acid, which can then go on to form DAG through the action of phosphatic acid phosphohydrolase (7). A more recently characterized signaling pathway involves the stimulation of a phosphocholine-specific phospholipase C activity which will produce increased levels of both DAG and phosphocholine in

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1 The abbreviations used are: PI, phosphoinositide; PLC, phospholipase C; PLD, phospholipase D; PC, phosphocholine; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; EGF, epidermal growth factor; Gi, inhibitory guanine nucleotide binding protein of adenyl cyclase; PDBu, phorbol 12,13-dibutyrate; DAG, 1,2-sn-diacylglycerol; PdBu, phosphatidylbutanol; PDGF, platelet-derived growth factor; PKC, protein kinase C (calcium/phospholipid dependent); 5-HT, 5-hydroxytryptamine (serotonin); TLC, thin layer chromatography; BSA, bovine serum albumin.
stimulated cells and has recently been demonstrated to provoke a mitogenic response (10–12).

We have recently observed that in a Chinese hamster lung fibroblast cell line (CCL39), a variety of agonists are capable of stimulating phosphoinositide-specific PLC activity and thus producing DAG. However, this stimulation rapidly desensitizes and does not correlate with a mitogenic response, suggesting that a short term activation of PKC does not convey enough information to allow cells to re-enter the cell cycle (13).

In this report, we examine the contribution of both phospholipase D and a PLC-specific phospholipase C to the mitogenic response. This was achieved by either agonist stimulation of each signaling pathway or by the addition of exogenous purified enzyme (PC-specific PLC). We demonstrate that the rate of PC hydrolysis by either a PLD or PLC enzymatic mechanism can be stimulated by both mitogenic and non-mitogenic stimuli which function through the activation of guanine nucleotide binding proteins (G-proteins). In contrast, ligands which exert their effects through receptors with intrinsic tyrosine kinase activity are incapable of stimulating either PLD or PLC. An exception to this is EGF, which is capable of stimulating PLD in a cell line that over-expresses the human EGF receptor.

Agonist-mediated stimulation of PLD was subject to a rapid desensitization, whereas stimulation of PC-PLC was found to occur over a long time frame. However, the hydrolysis of PC by exogenously added PC-PLC (Bacillus cereus) was not mitogenic on its own at any of the concentrations tested and induced cell death at high concentrations. In light of these findings, the importance of lipid breakdown to mitogenic signaling is discussed.

EXPERIMENTAL PROCEDURES

Materials

Highly purified human α-thrombin and recombinant basic FGF were generous gifts of Dr. J. W. Fenton II (New York State Departmen of Health, Albany, NY) and Dr. D. Gospodarowicz (University of California Medical Center, San Francisco, CA), respectively. [Methyl-3H]Thymidine, methyl-[3H]inositol, [9,10-3H]palmitic acid, [methyl-3H]choline chloride, and [γ-32P]ATP were obtained from Amersham Corp. All other materials were obtained from Sigma unless otherwise stated.

Methods

Cells and Culture Conditions—CCL39 cells are an established line of Chinese hamster lung fibroblasts (American Type Culture Collection). Cells expressing H1 receptor were obtained as described previously (13). Cells expressing the human EGF receptor were obtained as described previously (14).

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO), supplemented with 5.5% fetal calf serum (FCS), antibiotics (50 units/ml penicillin and 50 mg/ml streptomycin), and 25 mm sodium bicarbonate at 37 °C in a humid atmosphere (5% CO2, 95% air). To obtain quiescent cells arrested in the Go/G1 phase of the cell cycle, confluent cultures were incubated for 24 h in serum-free medium. When cells were pretreated with pertussis toxin, the medium (1:1) containing [3H]thymidine (3 μCi/ml, 24 h) was replaced by 2 ml of DMEM, 1% FCS, containing 4 μCi of [3H]palmitic acid. Prelabeling of the cells to near isotopic equilibrium occurred for 48 h. In certain experiments, PKC was down-regulated by pretreatment of the cells with 100 nM PDBu for the last 24 h of labeling. Prior to stimulation, cells were washed for 30 min with 1 ml of DMEM, 1% BSA. Cells were stimulated in this (freshly added) media with appropriate agonist or vehicle and 3 mm butan-1-ol. Stimulation was stopped by rapid aspiration of media followed by addition of 500 μl of methanol. Cells were scraped from the plate and collected in a glass vial. The plate was washed with a further 200 μl of methanol to give a final volume of 700 μl. Lipids were extracted by the addition of 700 μl of chloroform. After 15 min at room temperature, 585 μl of 1 M NaCl was added to each vial to give a final chloroform/methanol/aqueous ratio of 1:1:0.8. After centrifugation (5000 × g, 5 min), the upper phase was removed and the lower phase dried under vacuum before being resuspended in 100 μl of chloroform:methanol (19:1) and loaded onto Whatman LKDSF TLC plates. Plates were developed in the organic phase of isooctane:ethylacetate:acetic acidwater, 50:40:10:100. The peak corresponding to phosphatidylcholine (PC, Rf approximately 0.38) was identified using a Berthold TLC plate scanner. [3H]PtdBut was quantified by scraping from the plate and liquid scintillation counting.

For time course experiments, the cells were pretreated with 1 ml of DMEM, 1% BSA, 3 mm butan-1-ol for 10 min prior to addition of agonist or vehicle.

For desensitization experiments, agonists or vehicle in 1 ml of DMEM, 1% BSA were added to the cells at time 0, and cells were given a “pulse” of butan-1-ol (3 mM final concentration) at the appropriate time and the assay continued as normal.

Analysis of Release of Water-soluble Choline Metabolites—Separation of choline, phosphocholine, and glycerophosphocholine was performed exactly as described by Cook and Wakecam (15). Quiescent cells in 12-well plates prelabeled with [3H]choline chloride (2 μCi/ml, 24 h) were washed twice with HEPES-buffered DMEM (pH 7.4) followed by a 30-min wash in HEPES-buffered DMEM, 1% BSA. Cells were stimulated in 500 μl of the same media (freshly added). At the times indicated, the media were rapidly collected and 500 μl of methanol added, followed by 1 ml of chloroform. After a 15-min incubation, the phases were separated by centrifugation. The top phase was removed and 4 ml of water added before being passed over a 1-ml column of Dowex 50X8 H+ (200–400 mesh). The flow-through was reduced to an additional 5 ml of phosphatidylcholine/butanol (PtdBut, Rf approximately 0.38) was identified using a Berthold TLC plate scanner. [3H]PtdBut was quantified by scraping from the plate and liquid scintillation counting.

DAG Measurements—Confluent cells in 6-well plates were rendered quiescent by 24 h of serum starvation. Prior to stimulation, cells were washed twice in HEPES-buffered DMEM (pH 7.4) and resuspended in 500 μl of this medium. After a suitable time period, medium was rapidly aspirated and stimulation terminated by the addition of ice-cold methanol. After scraping the cells into a glass vial, lipids were extracted by the method of Bligh and Dyer (14). DAG levels were determined using the specific Escherichia coli DAG kinase kit obtained from Amersham Corp.

Protein Determinations—Assays were performed as described by Lowry et al. (17).

Data Presentation—Assays were performed in either duplicate or triplicate. The data presented are from representative experiments performed at least twice. Where mentioned, data from two or more experiments are pooled.

RESULTS

A continuing problem in the dissection of the signal transduction pathways required for mitogenesis is that of receptor heterogeneity, such that a particular agonist may activate a variety of signaling pathways through the activation of several receptor subtypes. To circumvent this problem, we have previously produced CCL39 clones which express high levels of either the human M1 muscarinic receptor (clone 39M1–81) (13) or the human EGF receptor (clone 39ER22) (14). These human receptors are either not expressed (M1 receptor) or
poorly expressed (EGF receptor) in parental CCL39 cells.

Stimulation of Phosphatidylcholine Hydrolysis in the 39M1–81 Clone—The hydrolysis of phosphatidylcholine (PC) by a phospholipase D results in the liberation of DAG along with phosphocholine. By contrast, PC hydrolysis by a phospholipase D produces phosphatidate along with choline (7, 11). Hence, in order to discern the mechanism of PC hydrolysis by both α-thrombin and carbachol in the 39M1–81 cell line, cells were firstly labeled to equilibrium with [3H]choline and choline metabolites separated by isoelectric chromatography as described under “Experimental Procedures.” Following the addition of either α-thrombin or carbachol, the level of both intracellular and extracellular [3H]choline was found to increase (Fig. 1, A and B). The increase in intracellular [3H]choline peaked 2 min after the addition of either agonist and was mirrored by a qualitatively similar increase in extracellular [3H]choline which was maximal after 5 min. In each case the response to carbachol was always greater than that elicited by α-thrombin. Interestingly, 30 min after the addition of each agonist, it was no longer possible to measure an increase in either intra or extracellular [3H]choline above basal levels. Under control conditions there was a marked increase in the level of [3H]phosphocholine in the extracellular media. Both carbachol and α-thrombin increased this accumulation, although the increase was not readily measurable until 20 min after addition of agonist (Fig. 1C). In contrast to the effects of both agonists on extracellular [3H]choline levels, the increased accumulation of [3H]phosphocholine was apparent for several hours after agonist addition. However, it was not possible to measure an agonist-induced increase in intracellular [3H]phosphocholine, even after long term stimulation (Fig. 1D). The reason for this is unclear, but it should be noted that the intracellular pool of [3H]phosphocholine is large and that the technique used may not be sufficiently sensitive to measure a comparatively small agonist-mediated increase. A second possibility is that the majority of PC-PLC activity is directed to the outer leaflet of the plasma membrane.

A variety of intracellular sources could theoretically contribute to the release of both [3H]choline and [3H]phosphocholine in the media of cells labeled with [3H]choline, the most obvious being the cytosolic pool of both compounds. It is formally possible that the agonist-induced increase of both [3H]choline and [3H]phosphocholine both intra- and extracellularly ([3H]choline) and extracellularly ([3H]phosphocholine) could arise from temporally distinct agonist-mediated phosphorylation/dephosphorylation events on both choline and phosphocholine in the cytosolic pool. However, we feel this is unlikely, since elegant studies in a fibroblastic cell line using a double labeling protocol with either [3H] or [3C] choline to selectively label either the lipid (long term labeling) or cytosolic (short term labeling) pool of choline metabolites have demonstrated that the source of released choline metabolites appears to be the choline-containing phospholipids rather than the cytosolic choline or phosphocholine pool (18, 19). We can thus propose that in CCL39 cells expressing the human M1 muscarinic receptor (39M1–81 cell line), α-thrombin and carbachol are capable of stimulating PC hydrolysis through both PLC and PLD enzymatic mechanisms.

The Production of DAG in 39M1–81 Cells in Response to α-Thrombin and Carbachol—In a variety of fibroblastic cell lines, DAG production has been demonstrated to be biphasic with a rapid peak of production often apparent in the 60-s period following agonist addition (20). This peak of DAG is thought to correspond to a transient increase in the level of PI-PLC activity (21). A more sustained phase of DAG production which may last several hours is then evident, which has been ascribed to hydrolysis of PC by either a PLC or PLD enzymatic mechanism (22, 22). In 39M1–81 cells, both α-thrombin and carbachol elicited a long term production of DAG which plateaued 15–20 min following agonist addition and remained elevated for at least 2 h (Fig. 2). Assuming that both agonists do not alter the rate of degradation of DAG, this suggests that DAG was being produced by long term activation of a lipid signaling pathway by both agonists, with carbachol being more potent in its stimulatory effect than α-thrombin. In some experiments, a rapid peak of DAG generation was apparent 30 s following addition of either agonist, but this was not reproducible (not shown). Unfortunately, the method used to quantitate DAG makes no distinction as to the exact composition of the DAG and hence the phospholipid source, although it would appear unlikely that the DAG is derived from either PI-PLC or from PLC-PD (see Ref. 13 and discussed later in text) due to the rapid desensitization of both pathways. An investigation into the ability of a variety of agonists (listed in Table I) to stimulate production of DAG
The ability of a variety of agonists to stimulate PI-PLC, PC-PLC, PLD, and DNA synthesis in 39M1-81 cells

The ability of the above ligands to stimulate the activity of PI-PLC was determined exactly as described under "Experimental Procedures" over a 5-min stimulatory period. Data are total inositol phosphates liberated ± range of duplicate determinations. DNA synthesis was determined by the ability of each agonist to stimulate the rate of incorporation of [3H]thymidine in cells rendered quiescent by whole cells by the addition of aluminium fluoride (AlF4-) to 39M1-81 cells elicits a modest stimulation of PI-PLC, PLD, and PC-PLC, suggesting that the three phospholipid hydrolyzing enzymes might be stimulated by a G-protein. Indeed in 39M1-81 cells the only agonist we have found which is capable of stimulating PC hydrolysis without stimulating PI hydrolysis is PDBu, this suggests that activation of PKC may directly stimulate both PC-PLD and PC-PLC activity. This suggests that ligands which are known to be either mitogenic or not, were screened for their ability to stimulate the rate of hydrolysis of either PC containing phospholipids by PLD (PC-PLD) or PLC (PC-PLC) or of inositol containing phospholipids (PI-PLC). In each case, a comparison was made with the mitogenic capability of each agonist, as measured by the ability to stimulate the incorporation of [3H]thymidine in quiescent cultures of 39M1-81 cells (Table I). It can be clearly seen that a strong correlation exists between the ability of an agonist to stimulate PI-PLC activity and the ability to stimulate the rate of hydrolysis of PC by both PC-PLD and PC-PLC. Indeed in 39M1-81 cells the only agonist we have found which is capable of stimulating PC hydrolysis without stimulating PI hydrolysis is PDBu, this suggests that activation of PKC may directly stimulate both PLD and PC-PLC, as will be discussed later. An additional major point revealed by the data in Table I is that there is apparently no correlation between the mitogenic capability of an agonist, as revealed by the ability to stimulate [3H]thymidine incorporation, and its ability to stimulate the rate of PC hydrolysis. Carbachol, which is poorly mitogenic (4.1% of the [3H]thymidine incorporation elicited by 10% serum), elicits a greater stimulation of PC-PLC and PC-PLD than does α-thrombin, a potent mitogen for this cell line. Of all ligands tested, PDBu elicits the greatest stimulation of PLD and PC-PLC but is poorly mitogenic in 39M1-81 cells (7% of the [3H]thymidine incorporation produced by 10% serum). Furthermore, ligands such as FGF and PDGF which exert their effect through binding to receptors which have intrinsic tyrosine kinase activity and are strongly mitogenic for this cell line (41 and 28%, respectively, of the [3H]thymidine incorporation elicited by 10% serum) have no measurable effect on the rate of each of PI-PLC, PC-PLD, or PC-PLC activity. This suggests that ligands such as FGF and PDGF do not exert their mitogenic effect through PI or PC signaling pathways and that these pathways are not essential for mitogenic signaling. Although G-proteins are normally activated by receptor stimulation, it is possible to circumvent the requirement for a receptor in whole cells by the addition of aluminum fluoride (AlF4-), which is thought to mimic the γ-terminal phosphate of GTP in the nucleotide binding site of the G-protein α-subunit and thus "fool" the G-protein into being active (26). Addition of AlF4- to 39M1-81 cells elicits a modest stimulation of PI-PLC, PLD, and PC-PLC, suggesting that the three phospholipid hydrolyzing enzymes may be stimulated by a G-protein(s) (results not shown). However, these data are not unequivocal, since AlF4- is capable of inhibiting phosphatase enzymes within the cell and the regulation of each of PI-PLC, PLD, and PC-PLC by phosphorylation/dephosphorylation events remains to be determined.

The proposed model of receptor activation by α-thrombin revealed that of all agonists tested, in addition to carbachol and α-thrombin, only serum, PDBu, and synthetic α-thrombin receptor activating peptide could maintain elevated DAG levels for over 45 min of agonist addition (results not shown). A possible problem with measurements of PC-PLD activity in whole cells is that the reaction products can be further metabolized, leading to underestimates of PLD activity. Hence, as a more definitive proof of a PLD-mediated reaction mechanism, we continued our studies on PLD-mediated PC hydrolysis in 39M1-81 cells by taking advantage of the trans-phosphatidylolation reaction. The cellular products of PC-PLD activity are phosphatidic acid and choline. However, in the presence of a primary alcohol such as n-butyl alcohol, the metabolically stable product phosphatidylbutanol is formed along with choline (7, 8). Dose-response studies indicated that the optimum concentration of n-butyl alcohol permissible was 0.3% final (v/v). At greater than 1% (v/v) n-butyl alcohol, cell viability was compromised and agonist-induced PLD stimulation reduced (results not shown).
Involvement of PLD and PC-PLC in Mitogenic Signaling

Involves the binding of α-thrombin to its receptor on CCL39 cells, followed by α-thrombin-mediated receptor cleavage at a specific extracellular site on the receptor and the formation of a new receptor N terminus (27, 28). The receptor is now in an active conformation either due to the release of an inbuilt inhibitory constraint or more likely by the production of an “autostimulatory” N-terminal region. This model implies that α-thrombin-mediated activation of its receptor is irreversible and that de-activation of the receptor requires a desensitization process. Evidence for the above described model of activation comes from experiments on CCL39 cells which demonstrate the ability of a synthetic peptide corresponding to the last 7 amino acids of the cleaved α-thrombin receptor (hamster) N-terminal to be able to entirely mimic most of the effects of α-thrombin on CCL39 cells. This is particularly evident regarding its ability to both stimulate phospholipid turnover by PI-PLC and to inhibit adenyl cyclase (29). Interestingly, the addition of this peptide to CCL39 cells results in the stimulation of both PLD and PC-PLC, in addition to the previously noted effects on both PI-PLC and adenyl cyclase (Table I). However, it should be noted that this peptide does not convey enough information to induce a mitogenic response (Ref. 24 and Table I) and that this inability is not due to a problem of peptide stability nor a difference in the ability of either α-thrombin or synthetic peptide to induce desensitization of the thrombin receptor.

Although demonstrating that the synthetic α-thrombin receptor peptide is incapable of fulfilling all the cellular functions of α-thrombin in CCL39 cells, this data also suggest that α-thrombin does not exert its mitogenic capability solely through modulation of adenyl cyclase, PI-PLC, PC-PLC, or PC-PLD activity.

Phospholipid Signaling in Cells Overexpressing Human EGF Receptors—In CCL39 cells, EGF receptors are expressed at a level of approximately 20,000 receptors/cell. In contrast to other ligands such as PDGF which bind to receptors with tyrosine kinase activity, EGF is not a potent mitogen in this cell line. We have previously investigated the mitogenic role of EGF in CCL39 cells transfected with and expressing high levels of the human EGF receptor (approximately 800,000 receptors/cell, 39ER22 cell line) (14). Interestingly, in 39ER22 cells, EGF is mitogenic, raising the possibility that EGF-mediated intracellular signaling in CCL39 cells is perhaps limited by receptor number (14). In direct contrast to results obtained with either CCL39 or 39M1–81 cells, in 39ER22 cells, EGF is capable of stimulating both the hydrolysis of inositol-containing phospholipids (Fig. 3b) in addition to stimulating PLD activity (Fig. 3a). However, both effects are small in comparison with that achieved with α-thrombin. Interestingly, the effect of EGF on both PI-PLC and PC-PLD are additive with the stimulation produced by 5-HT on both systems. It may be suggested that the effect of EGF on PI metabolism is due to an activation of PLC-γ, but this remains untested (30). In parallel with results obtained on CCL39 cells (Table I), it was not possible to demonstrate an effect of EGF on PC-PLC in 39ER22 cells, suggesting that activation of PC-PLC is not a direct consequence of either increased PI-PLC or PLD activity (results not shown). Although these data do not demonstrate that an increase in the rate of phospholipid turnover (PI-PLC and PLD) is necessary for an EGF-provoked mitogenic response, they suggest that the number of EGF receptors expressed in 39M1–81 cells limits the intracellular responses which can be measured.

The Time Course of PLD Stimulation by α-Thrombin and α-Thrombin, carbachol, and PDBu. 39M1–81 cells prelabeled with [3H]palmitate were stimulated with 1 unit/ml α-thrombin ( ), 10⁻⁴ M carbachol (○), or 10⁻⁵ M PDBu (●), and the formation of PtdBut was followed over time. In this experiment, cells were prewashed with 0.5% butan-1-ol 5 min before agonist addition to ensure that PtdBut formation at early time points was not limited by butan-1-ol availability. Error bars represent the range of duplicate determinations taken from an experiment representative of three performed.

Carbachol in CCL39 Cells—The results obtained in Fig. 1B demonstrate that agonist stimulation of intracellular PLD activity is rapid, with maximal activity obtained after approximately 2 min of agonist addition. To analyze the time course of PLD activation in more detail, we made use of the transphosphatidylation reaction (Ref. 7 and references therein). Cells were prewashed with n-butanol alcohol prior to agonist addition at time 0. After the appropriate period of incubation, the assay was stopped as detailed under “Experimental Procedures.” The stimulation of PLD activity by both α-thrombin and carbachol in 39M1–81 cells is rapid (Fig. 4), reaching a plateau after approximately 3 min of stimulation. Although the kinetics of activation of PLD by both agonists are similar, carbachol routinely gave a greater maximal stimulation. Similar experiments conducted using 100 nM PDBu as agonist revealed a similar plateau, although the maximal stimulation obtained was 1.5-fold greater than that obtained with car-
bacbol, and the initial rate of PLD stimulation in response to PDBu was apparently slower than that obtained with α-thrombin and carbachol. In several experiments, the basal PLD activity varied over a 2-fold range, whereas the agonist stimulation showed little variation. The plateau of stimulation is probably not a consequence of substrate limitation, since the quantity of PC approaches 50% of the total membrane lipid, and less than 0.001% of the pool has been hydrolyzed by carbachol after 5 min of stimulation (assuming that the majority of the pool is available to carbachol-stimulatable PLD activity). In addition, butan-1-ol is not limiting, since an additional pulse of butan-1-ol with agonist does not restore activity (results not shown). The [3H]PtdBut formed by PLD remains elevated at least up to 1 h after addition of agonist (Fig. 4). As PtdBut is not known to be further metabolized (31), this suggests that the plateau phase seen in response to α-thrombin, carbachol, and PDBu is as a result of PLD being no longer active.

Dose Dependence of PLD Stimulation—Dose-response studies on the activation of PLD by each of α-thrombin, carbachol, and PDBu were performed on 39M1-81 cells over a 10-min period (Fig. 5). The EC$_{50}$ for PLD activation by α-thrombin and carbachol in 39M1-81 cells was 3 × 10$^{-7}$ M and 3 × 10$^{-6}$ M, respectively, with maximal stimulation occurring with 3 × 10$^{-6}$ M α-thrombin and 10$^{-2}$ M carbachol. These values are in excellent agreement with those obtained for the stimulation of PI turnover in 39M1-81 cells by both α-thrombin and carbachol (Ref. 13 and results not shown). PDBu stimulates PLD activity with an EC$_{50}$ of 3 × 10$^{-7}$ M, maximal stimulation being obtained with 10$^{-7}$ M PDBu. In contrast to both α-thrombin and carbachol, PDBu does not stimulate PI turnover in 39M1-81 cells but instead is capable of inhibiting receptor-mediated stimulation of this enzyme, possibly through an as yet undefined negative feedback mechanism (see below and Ref. 33).

The Role of PKC in PLD Stimulation in 39M1-81 Cells—Based on the results shown in Table I and in Figs. 3 and 5, there is apparently a strong correlation between the ability of an agonist to stimulate PI-PLC and the ability to regulate PLD in a positive manner. The exception to this rule is PDBu, a direct stimulator of PKC (2, 6) and a strong stimulator of PC breakdown. A direct consequence of PI-PLC activation is the concomitant activation of PKC (1, 2), we thus reasoned that activation of PC breakdown is perhaps a response secondary to the stimulation of PI-PLC. The role of PKC in PC breakdown was examined by pretreatment of 39M1-81 cells for 24 h with 200 nM PDBu. This represents a concentration of PDBu sufficient to elicit a complete loss of PKC activity (32). After such pretreatment, the ability of α-thrombin, carbachol, and PDBu to stimulate PLD activity was almost completely attenuated (Fig. 6a). Additionally, after a similar treatment of 39M1-81 cells with PDBu, α-thrombin and carbachol were no longer able to stimulate PC-PLC activity (results not shown). Interestingly, PKC down-regulation did not attenuate the ability of both α-thrombin and carbachol to stimulate PI-PLC activity (Fig. 6b). Rather, the initial rates of PI hydrolysis in response to both agonists were greater after PDBu-mediated down-regulation of PKC. This may result from a loss of negative feedback by PKC on PI-PLC, as mentioned above and as has been documented previously (33).

G-protein Involvement in Phospholipid Hydrolysis; the Effect of Pertussis Toxin—The mitogenic action of α-thrombin is almost completely abolished by pretreatment of both CCL39 or 39M1-81 cells with pertussis toxin, thus defining an obligate role for one or more pertussis toxin-sensitive G-proteins in mediating the response (34). CCL39 cells express at least four such G-proteins, Gia2, Gia3, Goa2, and an as yet undefined Goa subtype which is not Goa1. An agonist-mediated increase in the rate of PC hydrolysis has been reported previously to be G-protein-mediated (35–37). Hence to establish the possible involvement of such a pertussis toxin-sensitive G-protein in the activation of both PLD and PC-PLC, 39M1-81 cells were treated for the last 4 h of preculture labeling with 100 ng/ml pertussis toxin, a concentration of toxin previously shown to completely ADP-ribosylate the entire pool of pertussis toxin-sensitive G-proteins within this

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F. R. McKenzie and J. Pouyssegur, manuscript in preparation.
time period (38). This treatment was found to inhibit the α-thrombin-mediated stimulation of PLD by approximately 30%, while having no effect on either the carbachol- or PDBu-mediated stimulation of the enzyme (Table II). These results are in absolute agreement with the pattern of pertussis toxin sensitivity of PI-PLC stimulation by both α-thrombin and carbachol in this cell line and demonstrate that the receptors for α-thrombin and carbachol (Hm1) do not use an identical pool of G-proteins to stimulate PI-PLC and PLD (13). Basal PLD activity was routinely increased by pertussis toxin pretreatment, albeit a minor effect. The reason for this is unclear. By contrast, neither basal activity nor stimulation of PC-PLC in response to α-thrombin, carbachol, or PDBu was affected by pertussis toxin pretreatment, suggesting that activation of both PLD and PC-PLC are mediated, at least in the case of α-thrombin, by nonidentical pathways.

Stimulation of PLD by α-Thrombin and Carbachol Rapidly Desensitizes—The ability of an agonist to induce the reinitiation of DNA synthesis in quiescent cells depends not only on the initial response of second messenger systems measured shortly after agonist addition, but on the activities persisting for several hours after the addition of growth factor (39, 44). The data presented in Figs. 1 and 4 suggest that although the activation of PC-PLC persists over at least several hours following agonist addition, the activation of PLD is more transient in nature. To examine the time course of stimulation of PLD in response to either α-thrombin, carbachol, or PDBu, each agonist was added to prelabeled 39M1–81 cells at time 0. Cells were then given a pulse of n-butyl alcohol at various times and the assay continued as normal (Fig. 7). It can thus be seen that the stimulation of PLD in response to both α-thrombin and carbachol rapidly desensitizes. Essentially no stimulation of PLD is evident 2 min following addition of agonist. Thus in the assay conditions employed, receptor-mediated stimulation of PLD is subject to a complete desensitization. We have shown previously that the stimulation of PI-PLC by α-thrombin and carbachol desensitizes and that this process occurs with a similar time course (13, 40). However, α-thrombin- and carbachol-mediated stimulation of PI-PLC do not fully desensitize. The stimulation of PLD by PDBu is also subject to a desensitization process, although the time course is somewhat slower than that observed for both α-thrombin and carbachol. This may be a reflection that more than one mechanism of PLD desensitization exists (Fig. 7).

Agonist-mediated desensitization of a receptor-mediated

**Table II**

<table>
<thead>
<tr>
<th>Pertussis toxin sensitivity of PLD and PC-PLC in response to carbachol and α-thrombin in 39M1–81 cells</th>
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<td>PLD activity was assayed in response to either carbachol (10^{-4} M), α-thrombin (1 unit/ml), or PDBu (100 nM) for 5 min in either control cells or cells pretreated with 100 ng/ml pertussis toxin for 4 h prior to agonist addition. We have demonstrated previously that this treatment fully ADP-ribosylates the complement of pertussis toxin sensitive G-proteins expressed in 39M1–81 cells (38). PLD-PC-PLC was assessed during a 30-min period in response to either carbachol (10^{-4} M), α-thrombin (1 unit/ml), or PDBu (100 nM) in either control or pertussis toxin-pretreated cells exactly as described in the legend to Fig. 1. Data are means ± S.D. of quadruplicate determinations pooled from two separate experiments.</td>
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<tr>
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<td>Control</td>
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<td>PDBu</td>
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**Fig. 7.** Desensitization of α-thrombin- and carbachol-induced PLD activity in 39M1–81 cells. Cells were stimulated with either 1 unit/ml thrombin (O), 10^{-4} M carbachol (□), or PDBu (100 nM) (●) at time 0. Measurement of PLD activity was achieved by giving a pulse of butan-1-ol (0.5%) at the times indicated, and the assay was continued for a further 6 min, after which time the amount of PtdBut produced was determined. In the absence of agonist, the PLD activity remained constant over the period of measurement.

**Fig. 8.** Receptor-mediated desensitization of PLD activity is heterologous with respect to carbachol but partially homologous with respect to both α-thrombin and α-thrombin receptor peptide. 39M1–81 cells prelabeled with [3H]palmitate were pre-treated with either vehicle (empty bars), α-thrombin at 1 unit/ml (black bars), carbachol at 10^{-4} M (striped bars), or α-thrombin receptor peptide at 100 nM (stippled bars) for 15 min. Cells were then rapidly washed and challenged with the agonists noted on the axis at the following concentrations: α-thrombin, 1 unit/ml; carbachol, 10^{-4} M; α-thrombin receptor peptide, 100 nM; PDBu, 100 nM; AlF4-, 1 mM. PLD activity was measured over a 5-min period following rechallenge with agonist. Data are means ± S.D. for triplicate determinations taken from an experiment representative of three others performed. All data represent increases above basal activity (no agonist) which was 1023 ± 23 cpm of [3H]phosphatidylbutanol.

response may be broadly classified into being of a homologous (only the response to the original agonist is attenuated) or a heterologous (the response to any agonist has been attenuated) nature (41, 42). In the case of receptor-mediated desensitization of PLD stimulation, the desensitization may be a result of modification of either the effector itself (PLD enzyme) of an intermediary G-protein or of only the receptor. Hence to further examine the mechanism of desensitization of the PLD response, 39M1–81 cells were split into four groups and were given no addition or were stimulated with either α-thrombin, carbachol, or α-thrombin receptor peptide for 15 min. After this time, the cells were challenged with either α-thrombin, α-thrombin receptor peptide, carbachol, PDBu, or AlF4− (Fig. 8). After treatment with carbachol, PLD is no longer able to respond to further challenge with either α-thrombin, α-thrombin receptor peptide, carbachol, or AlF4−, but it retains the ability to be stimulated by PDBu. In contrast, in cells pretreated with α-thrombin, PLD is refractory to rechallenge with both α-thrombin and α-thrombin recep-
tor peptide but may be stimulated with carbachol, AlF₄⁻, and PDBu. It should be noted that the ability of both carbachol and AlF₄⁻ to stimulate PLD are reduced by 30 and 31%, respectively, after α-thrombin pretreatment. After pretreatment with α-thrombin receptor peptide, it is no longer possible to stimulate PLD with either receptor peptide or α-thrombin, although PDBu-mediated stimulation of PLD is not altered. Carbachol and AlF₄⁻ are both still able to stimulate PLD after α-thrombin receptor pretreatment, although the magnitude of stimulation is reduced by 34 and 33%, respectively. This demonstrates that receptor-mediated desensitization of PLD stimulation is heterologous with regard to carbachol and partially homologous with regard to both the α-thrombin and α-thrombin receptor peptide. The reason for this difference in the desensitization process is not immediately apparent and will be discussed later.

**Time Course of Recovery of Receptor-mediated PLD Stimulation after Treatment with α-Thrombin or Carbachol**—In view of the results shown in Fig. 8, we decided to examine the time course of re-sensitization of agonist-stimulated PLD activity in 39M1-81 cells. This was achieved by pretreating cells which had been prelabeled to isotopic equilibrium with [³²⁵]IPalmitate with either α-thrombin, α-thrombin receptor peptide, or carbachol for 15 min. After agonist pretreatment, the cells were either washed repeatedly at 37 °C to remove agonist (carbachol-pretreated cells), or in the case of α-thrombin pretreated cells, a 10-fold excess of hirudin was added to the cells for 5 min, followed by repeated washing at 37 °C to remove free hirudin and α-thrombin-hirudin complexes. The latter treatment has been shown previously to effectively remove α-thrombin from extracellular binding sites (39). We could thus be sure that agonist was no longer present. Following pretreatment of 39M1-81 cells with carbachol (Fig. 9a), it was no longer possible to stimulate PLD by either α-thrombin or carbachol (time 0). However, after carbachol removal, the ability of both carbachol and α-thrombin to stimulate PLD rapidly recovered, the response being fully restored 30 min after removal of the desensitizing agonist. A similar recovery of the ability of AlF₄⁻ to stimulate PLD activity was also obtained (results not shown). In contrast, as previously noted (Fig. 8), after pretreatment of cells with α-thrombin (Fig. 9b), although further challenge with α-thrombin was unable to stimulate PLD, the ability of both carbachol and AlF₄⁻ to stimulate PLD was retained (results not shown). Restoration of the ability of α-thrombin to stimulate PLD required 16 h of protein synthesis as evidenced by the lack of re-sensitization to α-thrombin in the presence of the protein synthesis inhibitor cycloheximide (Fig. 9b). It is therefore evident that α-thrombin-induced desensitization of its receptor, as measured by the ability to stimulate PLD activity, is complete and requires new receptors to restore the response. By contrast, carbachol-mediated desensitization of the human M₁ receptor is incomplete, and the ability to stimulate PLD quickly recovers.

**Extracellular Addition of PC-PLC Hydrolyzing Enzyme and Its Effect on PC-PLC Hydrolysis and Mitogenic Signaling**—A recent report has demonstrated that exogenous addition of a purified PC-PLC-specific enzyme from *B. cereus* can serve as a mitogenic signal for Swiss 3T3 fibroblasts (12, 21). In 39M1-81 cells both mitogenic (α-thrombin) and non-mitogenic (carbachol) agonists have been shown to elicit a long term activation of PC-PLC (Fig. 1), although carbachol is not a mitogen for this cell line. This provides strong evidence against a dominant role for PC-PLC in mitogenic signaling. However, to further analyze the contribution of PC-PLC in mitogenic signaling, the effects of exogenously added PC-PLC-specific enzyme (source *B. cereus*, Sigma) on PC hydrolysis were examined. In 39M1-81 cells prelabeled with [³²⁵]IPalmitate, exogenously added PC-PLC was able to release large amounts of [³²⁵]Iphosphocholine in a dose-dependent manner (Fig. 10a). At the maximal concentration used (10 units/ml), greater than 14% of the radiolabeled lipid pool was liberated following a 40-min stimulatory period (compare with the maximal effect shown by carbachol, Fig. 1). This stimulatory effect did not appear to follow a normal dose-response curve, but leveled off, most probably due to kinetic constraints of substrate limitation (results not shown). As was expected, a 24-h pretreatment of the cells with PDBu to down-regulate PKC had no effect on the ability of *B. cereus* PC-PLC to liberate phosphocholine (results not shown). The effect of PC-PLC on DNA reinitiation is shown in Fig. 10b. When added on its own to CCL39 cells, exogenously added PC-PLC has no discernable effect on the incorporation of [³²⁵]Ithymidine. However, when added together with FGF, PC-PLC is able to synergize in a dose-dependent manner. At concentrations of PC-PLC greater than 0.1 unit/ml, the incorporation of [³²⁵]Ithymidine is massively reduced, this is a result of cell death produced by the enzyme. These results indicate that addition of PC-PLC to CCL39 cells does not in itself constitute a mitogenic response but that the increase in PC breakdown can synergize with known growth factors.
Involvement of PLD and PC-PLC in Mitogenic Signaling

In this study, we have analyzed the role of PC-PLC in response to a variety of stimuli. The latter has proved fruitful in that we have previously been able to demonstrate that the mitogenic effect of α-thrombin in CCL39 cells is not mediated by agonist receptors but may be adopted in either comparing the signaling pathways activated, although α-thrombin is known to modulate the level of activity of PKC, an assumption which may not be entirely correct (45, 47, 48).

At present, the exact signal transduction pathway(s) by which α-thrombin stimulates mitogenesis remains to be elucidated, although α-thrombin is known to modulate the activity of an apparently limited number of intracellular effectors via activation of both pertussis toxin-sensitive and insensitive G-proteins (43). A simplistic approach which may be adopted is to either compare the signaling pathways activated by α-thrombin and a variety of both mitogenic and non-mitogenic stimuli or to attempt to "reconstitute" the mitogenic-signaling pathway. The latter has proved fruitful in that we have previously been able to demonstrate that the mitogenic effect of α-thrombin in CCL39 cells is not mediated by this hormone (13). However this did not rule out the involvement of some of the more recently described receptor-activated lipid signaling pathways such as the hydrolysis of PC phospholipids. In this study, we have analyzed the role of PC breakdown in mitogenic signaling in response to a variety of different stimuli: (a) agonists which function through G-protein activation and are mitogenic-typhied by α-thrombin; (b) agonists which function through G-protein activation and are not mitogenic, typhied by carbachol (human M1 muscarinic receptor); (c) agonists which are mitogenic and function through the activation of a receptor with intrinsic tyrosine kinase activity, examples being FGF, PDGF, and EGF; (d) exogenous addition of purified PC-PLC enzyme from B. cereus.

Using the above classes of agonists the data presented in this study clearly demonstrate that in the cell lines under study, an increase in the rate of PC hydrolysis by either a PLC or a PLD enzymatic mechanism is neither sufficient on its own, nor essential to mitogenic signaling.

It may be stated that a major requirement for a signaling pathway which plays a role in mitogenesis is that the pathway remains active for many hours following agonist addition (39, 43, 44). The activation of protein kinase C has been shown to play a role in mitogenic signaling (2, 4–6). However, at present, measurements of PKC activity in response to growth factor stimulation have only been made acutely (up to 30 min following agonist addition) (45). This is in part due to current methods of measuring PKC activity in plasma membrane fractions which examine total stimulatable activity in the membrane at a given time rather than the actual stimulated activity. In addition, after translocation to the plasma membrane, PKC may follow a complex mechanism of activation requiring proteolytic cleavage of the enzyme to produce a constitutively active PKC moiety (46). Hence we felt that the enzyme was being continuously stimulated.

It is acknowledged that activation of PKC requires increased diglyceride levels, hence we reasoned that an increase in DAG may be taken as an index of increased PKC activity (47, 48). The diglyceride, DAG, may be produced by the hydrolysis of a variety of cellular phospholipids, including phosphatidylinositol 4,5-bisphosphate, the preferred substrate of PI-specific PLC (1). We have reported previously that agonist-mediated stimulation of PI-PLC rapidly desensitizes, suggesting that activation of PKC may be extremely transient in nature. However, continued hydrolysis of PC by either PLD- (followed by the action of phosphatidic acid phosphohydrolase) or by PLC-specific PLD could theoretically provide an alternative pathway of lipid hydrolysis to maintain elevated DAG levels (7–9). An examination of DAG levels in 39M1–81 cells in response to both α-thrombin and carbachol reveals that both agonists can increase the intracellular DAG level over a period of several hours (Fig. 2). Thus it may be possible that both agonists elicit the continual stimulation of PKC. However this is based on the assumption that DAG derived from different membrane phospholipids is equally able to stimulate PKC, an assumption which may not be entirely correct (45, 47, 48).

In 39M1–81 cells, an agonist-mediated increase in the rate of hydrolysis of PC proceeds by both PLC- and PLD-mediated mechanisms. However a major difference is evident in the duration of each response. Receptor-mediated hydrolysis of PC by PLD by both mitogenic stimuli such as α-thrombin and non-mitogenic stimuli such as carbachol is subject to a rapid desensitization such that no further PLD activity on PC is detectable 5 min following agonist addition. This allows us to conclude that the stimulation of PC-PLD is not required for the mitogenic effect of α-thrombin. In contrast to PC-PLD, a receptor-mediated increase in the level of activity of PC-PLC is not subject to a desensitization process over a period of 4 hours following agonist addition (not shown). As already mentioned, a signal pathway involved in mitogenesis should be continually active to a certain extent, in the presence of agonist. Hence it would appear that receptor-stimulatable PC-PLC fulfils this obligation. However it should be
noted that the non-mitogenic agonist carbachol is a more potent activator of PC-PLC than α-thrombin and that the α-thrombin receptor peptide is at least as potent as α-thrombin in its ability to stimulate PC-PLC, suggesting that stimulation of PC-PLC activity is not sufficient for α-thrombin's mitogenic capability. At present, our data suggest that the sustained increase in DAG levels in 39M1–81 cells in response to both α-thrombin and carbachol cannot be accounted for by either PI-PLC or PC-PLD activity but is perhaps emanating from continual PC-PLC activity.

Further evidence to dispel the idea that an increase in the rate of hydrolysis of PC phospholipids can serve as a mitogenic signal comes from experiments conducted with purified PC-specific PLC enzyme from B. cereus. Previous reports have suggested that an increase in the rate of PC hydrolysis by PC-specific PLC is important in cell proliferation (9, 12, 24, 25) and may constitute part of the mitogenic signal elicited by PDGF in Swiss 3T3 cells (12). However the kinetics of PC-PLC stimulation by PDGF were exceptionally slow, requiring a period of 4 h before the effect of PDGF was measurable. This of course makes it impossible to correlate a PDGF-mediated increase in PC hydrolysis with an early receptor-mediated event (12). The same group has also demonstrated that the addition of purified PC-PLC enzyme to cultures of Swiss 3T3 fibroblasts can serve as a mitogenic signal (12). Similar experiments performed on either 39M1–81 cells or the parental CCL39 cell line demonstrate that although addition of purified PC-PLC elicits a massive increase in the rate of PC hydrolysis, this does not in itself serve as a mitogenic signal.

Both α-thrombin and carbachol function through the activation of G-proteins. In the case of thrombin, mitogenic signaling is entirely dependent on pertussis toxin-sensitive G-proteins (38), defining a role for a signal transduction pathway modulated by α-thrombin which is mediated by one or several of the four pertussis toxin-sensitive G-proteins (Gi2, Gi3, Goα1, and GoαX) expressed by CCL39 cell and derivatives. Considerable evidence has demonstrated that hydrolysis of PC is under the control of G-proteins, although it is debatable whether G-proteins interact directly with either PC-PLD or PC-PLC (7). Although several reports have demonstrated activation of PLD that appears to be independent of PKC (31, 49), our experiments have demonstrated an obligate role for PKC in the stimulation of PC-PLD and PC-PLC, such that it is no longer possible to stimulate either enzyme in cells depleted of PKC by long term phorbol ester treatment. It is possible that PKC down-regulation data is misinterpreted in that long term phorbol ester treatment not only down-regulates PKC, but also PLD. If this is indeed the case, then there may be a PKC-independent pathway of PLD activation which is no longer visible after long term phorbol ester treatment. Definitive proof awaits either PLD-specific antibodies or reconstitution methods to measure PLD activity (50). Interestingly, stimulation of PKC is not mitogenic in CCL39 cells or their variants, and down-regulation of PKC does not inhibit the mitogenic effect of α-thrombin (13). This provides further evidence to dispel a role for PLD activity in α-thrombin-mediated mitogenesis.

The mechanism of desensitization of PLD in 39M1–81 cells shows marked differences depending on the stimulating agonist. If α-thrombin is the desensitizing agent, then PLD remains refractory for up to at least 4 h to further stimulation by α-thrombin. However, after a short time lag (approximately 15 min), it is possible to fully stimulate PLD with a different agonist, carbachol. Identical results are obtained if α-thrombin receptor peptide is used instead of α-thrombin (results not shown). By contrast, if carbachol is the desensitizing agonist, the ability of both α-thrombin and carbachol to stimulate PLD is fully restored after a similar time lag. Thus although the PLD signaling system has the capacity to be re-stimulated after a short recovery period, the receptor for α-thrombin does not recover. The inability of the α-thrombin receptor to respond to a second challenge with agonist is a consequence of the mechanism of activation of this receptor (27, 28). Upon α-thrombin binding, the thrombin receptor is cleaved, leaving a new N-terminal tail which has lost 41 amino acids. The new receptor N terminus can now interact with another region(s) of the receptor, thus promoting an active conformation. This implies that after cleavage of the α-thrombin receptor, agonist (the receptor's N-terminal region) will be continually present and not removable. In this scenario, if the cell wishes to attenuate the signal coming directly from the active α-thrombin receptor, there are a variety of known choices: desensitization of the receptor, presumably by a modification such as phosphorylation (41, 42); down-regulation of the receptor (51); or modification of the signaling components distal to the receptor (52). Activation of the α-thrombin receptor by either α-thrombin (receptor cleavage) or by a synthetic peptide corresponding to the N-terminal region of the cleaved receptor results in the activation and desensitization of the receptor, as evidenced by the inability of the receptor to recover its ability to stimulate PLD after agonist removal and a recovery period of several hours. In contrast, after carbachol-mediated desensitization of the M1 muscarinic receptor, full recovery of the ability to stimulate PLD is obtained 30 min after the removal of agonist. It would thus appear that the inability of α-thrombin to restimulate PLD activity is not due to a refractoriness of PLD itself, but more likely due to the loss of functioning α-thrombin receptors on the surface of the cell. Interestingly, identical results are obtained when one examines the desensitization of α-thrombin's ability to inhibit adenylyl cyclase. Complete recovery of the ability of α-thrombin to stimulate PLD requires at least 16 h of protein synthesis, suggesting that the desensitization of the α-thrombin receptor is irreversible. For receptors where desensitization phenomena have been studied in detail, such as the β-adrenergic receptor, it is apparent that desensitization is followed by a more slowly occurring down-regulation of the receptor (41, 42). However, we are at present unable to assess whether the loss of α-thrombin responsiveness following α-thrombin pretreatment is as a result of both desensitization and/or down-regulation, since it is not possible at present to perform ligand binding studies. It could be hypothesized that the rapid recovery of the M1 muscarinic receptor after desensitization with carbachol is not due to the desensitization being reversible, but due instead to the rapid turnover of the M1 receptor. This question remains to be examined.

We have demonstrated previously that the stimulation of PLC by each of α-thrombin and carbachol is subject to a rapid albeit incomplete desensitization (19). This desensitization is entirely homologous in nature. However in the case of PLD, carbachol mediates a heterologous form of desensitization, such that after carbachol pretreatment, the ability of each of α-thrombin, α-thrombin receptor peptide, and Aβ42 to activate the enzyme is reduced. This suggests that carbachol, in addition to promoting desensitization of the M1 muscarinic receptor, is modifying more distal components in the signal transduction cascade. PLD itself does not appear to be modified, since there is no apparent reduction in the ability of PDBu to stimulate PLD after a pretreatment with carbachol. Interestingly, qualitatively identical data are obtained
when one performs a similar series of experiments and
examines the ability of both α-thrombin and carbachol to liberate
Ca\textsuperscript{2+} from intracellular stores in 39M1–81 cells.\textsuperscript{4} After
α-thrombin pretreatment, α-thrombin is no longer able to
liberate intracellular Ca\textsuperscript{2+}, whereas the carbachol-induced
release of DAG may still play a role in the mitogenic response.
Indeed we have recently found that the persistent phase of
MAP kinase activation correlates very well with the mitogenic
capability of α-thrombin in cells.\textsuperscript{4} After a relatively
long term activation of PC-PLC with the concomitant gen-
eration of DAG may still play a role in the mitogenic
response. This phase of activation is multifactorial, one factor
potentially sensitive coupling to the latter has recently been
demonstrated that the α-thrombin-mediated mitogenic effect involves the activation of one or more per-
tussin toxin-sensitive G-proteins. A continuing question is the

2. P., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, D.,

3. Garcia de Herreros, A., Dominguez, I., Diaz-Meco, M. T., Graziani, G.,


12. Larrodera, P., Cornet, M. E., Diaz-Meco, M. T., Lopez-Barahona, M.,


Factors 1, 311-321


23. Garcia de Herreros, A., Dominguez, I., Diaz-Meco, M. T., Graziani, G.,


25. Lopez-Barahona, M., Kaplan, P. L., Cornet, M. E., Diaz-Meco, M. T.,


29. Rasmussen, U. B., Vouret-Craviari, V., Jallet, S., Schlesinger, Y., Pages, G.,


31. Vouret-Craviari, V., Van Obberghen-Schilling, E., Rasmussen, U., Pavirani, A.,


