Assessment of receptor-dependent activation of phosphatidylcholine hydrolysis by both phospholipase D and phospholipase C

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Enhancement of cellular phospholipase D (PLD)-¹ and phospholipase C (PLC)-mediated hydrolysis of endogenous phosphatidylcholine (PC) during receptor-mediated cell activation has received increasing attention inasmuch as both enzymes can result in the formation of 1,2-diacylglycerol (DAG). The activities of PLD and PLC were examined in purified mast cells by quantitating the mass of the water-soluble hydrolysis products choline and phosphorylcholine, respectively. Using an assay based on choline kinase-mediated phosphorylation of choline that is capable of measuring choline and phosphorylcholine in the low picomole range, we quantitated the masses of both cell-associated and extracellular choline and phosphorylcholine. Activating mast cells by crosslinking its immunoglobulin E receptor (Fcε-RI) resulted in an increase in cellular choline from 13.1 ± 1.2 pmol/ 10^6 mast cells (mean $\pm$ SE in unstimulated cells) to levels 5- to 10-fold higher, peaking 20 s after stimulation and rapidly returning toward baseline. The increase in cellular choline mass paralleled the increase in labeled phosphatidic acid accumulation detected in stimulated cells prelabeled with [³H]palmitic acid and preceded the increase in labeled DAG. Although intracellular phosphorylcholine levels were ~15-fold greater than choline in unstimulated cells (182 ± 19 pmol/ 10^6 mast cells), stimulation resulted in a significant fall in phosphorylcholine levels shortly after stimulation. Pulse chase experiments demonstrated that the receptor-dependent increase in intracellular choline and the fall in phosphorylcholine

were not due to hydrolysis of intracellular phosphorylcholine and suggested a receptor-dependent increase in PC resynthesis. When the extracellular medium was examined for the presence of water-soluble products of PC hydrolysis, receptor-dependent increases in the mass of both choline and phosphorylcholine were observed. Labeling studies demonstrated that these extracellular increases were not the result of leakage of these compounds from the cytosol. Taken together, these data lend support for a quantitatively greater role for receptor-mediated PC-PLD compared with PC-PLC during activation of mast cells.

Introduction

Receptor stimulation in a variety of cells is associated with the hydrolysis of phosphatidylcholine (PC) by a variety of cellular phospholipases (reviewed by Exton, 1990). One aspect of the importance of PC hydrolysis lies in the ability of phospholipase C (PLC) to form 1,2-diacylglycerols (DAGs)², which importantly contribute to the regulation of cell activation by modulating the activity of protein kinase C (PKC) (reviewed by Kikkawa and Nishizuka, 1986). In addition to the role of DAG as a second-messenger molecule, increasing biophysical evidence points to a potentially critical role for DAG in the development of membranous intermediates that promote membrane fusion during exocytosis (Das and Rand, 1984; Siegel *et al.*, 1989).

While attention has been focused primarily on the activity of phosphatidylinositol (PI)-PLC in the formation of DAG, increasing evidence points to an important role for PC as substrate in DAG formation (reviews by Kennerly, 1989; Exton, 1990). In addition to the direct formation of DAG by PLC, increasing evidence indicates that DAG may be produced by the sequential

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¹ Abbreviations used in this paper: DAG, 1,2-diacylglycerol; Fcε-RI, the high-affinity receptor for immunoglobulin E (IgE); PA, phosphatidic acid; PA-PHase, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; TPB, tetraphenylboron.

² In the current manuscript the more specific term DAG has been used rather than the term diacylglycerol because, in contrast to other cells, the rat serosal mast cell does not contain significant 1,*O*-alkyl- or alk-1-enyl- forms of PC, PE, or diglycerides (Kennerly, 1987a, 1990).

actions of phospholipase D (PLD) and phosphatidic acid phosphohydrolase (PA-PHase) in the indirect pathway illustrated in Figure 1 (Bocckino *et al.*, 1987; Cabot *et al.*, 1988; Pai *et al.*, 1988; Agwu *et al.*, 1989; Billah *et al.*, 1989; Liscovitch and Amsterdam, 1989; Gruchalla *et al.*, 1990).

Evidence supporting receptor-initiated increases in PLC-catalyzed PC hydrolysis has been generated in a number of laboratories. With the use of a variety of model systems, cells in which PC has been prelabeled with radioactively-labeled choline have been shown to release labeled phosphorylcholine either into the extracellular medium or into the cytosol after appropriate stimulation (Besterman *et al.*, 1986; Daniel *et al.*, 1986; Cabot *et al.*, 1988; Slivka *et al.*, 1988; Martinson *et al.*, 1989).

That receptor-dependent, PLD-mediated hydrolysis of PC occurs during cell activation is indicated by a variety of experimental approaches. In early work, cellular PC was prelabeled by [³H]choline, and, in chase experiments, the labeled choline was found to be liberated from PC, as evidenced by rises in extracellular choline after cellular stimulation, usually by the PKC-activating phorbol esters (Mufson *et al.*, 1981; Daniel *et al.*, 1986; Takuwa *et al.*, 1987; Cabot *et al.*, 1988; Slivka *et al.*, 1988). Experiments seeking to detect an increase in the generation of phosphatidic acid (PA; the hydrophobic product of PLD-mediated phospholipid hydrolysis) have used both fatty acid labeling strategies (Cabot *et al.*, 1988; Pai *et al.*, 1988; Agwu *et al.*, 1989; Billah *et al.*, 1989; Liscovitch and Amsterdam, 1989; Gruchalla *et al.*, 1990) and mass analysis (Bocckino *et al.*, 1987). In most experiments the level of labeled PA or PA mass was found to rise with kinetics more rapid than that of DAG, suggesting that PA was more likely to act as a substrate in the formation of DAG than as the product of DAG phosphorylation. Molecular species or fatty acid analysis of labeled PA or PA mass, similarly, have shown that PA bears a striking similarity to that of the DAG found to accumulate as a result of stimulation (Cockcroft and Allan, 1984; Bocckino *et al.*, 1987). Billah's group (Billah *et al.*, 1989) used a doubly labeled precursor that resulted in the intracellular accumulation of 1-O-[³H]alkyl,2-acylglycerol[³²P]phosphorylcholine to show that the accumulation of PA was initially due to the activation of PLD rather than to the sequential actions of PLC and DAG kinase. In similarly elegant experiments, Bocckino and Exton (Bocckino *et al.*, 1985, 1987; Irving and Exton, 1987) have demonstrated not only the accumulation

of PA mass in hepatocyte plasma membranes after stimulation by vasopressin but also the likely regulation of PLD activity by guanine nucleotide-binding proteins.

Although these studies using endogenously labeled PC are of considerable value in suggesting the activation of PLC and/or PLD, determination of the mass of choline and phosphorylcholine would more clearly estimate the magnitude of these activities, particularly in terminally differentiated cells in which uniform prelabeling is not possible. Truett *et al.* (1989) presented a preliminary report of a sensitive choline assay and findings indicating that intracellular choline levels in neutrophils increase with stimulation, whereas levels of phosphorylcholine are modestly reduced. In collaborative studies we have modified this technique for greater sensitivity and ease and extended it to phosphorylcholine (Murray *et al.*, 1990). Bocckino *et al.* (1987) demonstrated that stimulation of hepatocyte plasma membranes by vasopressin caused an increase in choline mass but no change in that of phosphorylcholine. They did not examine changes in intact cells, however, or assess whether hydrolysis occurred on the cytosolic side of the plasma membrane. In the current report we describe the use of this mass-based choline assay to examine the mass of both intracellular and extracellular choline metabolites in mast cells. Our findings strongly suggest the importance of receptor-dependent activation of cellular PC-PLD.

Results and discussion

Receptor-mediated formation of PA and DAG

Because methods are not sufficiently sensitive to consistently and accurately detect the mass of PA from 10⁶ mast cells (the total yield from 1 rat), the receptor-dependent formation of PA from other lipids was examined by prelabeling mast cells with the fatty acid, [³H]palmitic acid. Total phospholipid labeling varied from 0.6 to 2.5 × 10⁶ CPM/10⁶ cells, but the fractional distribution of label in the different phospholipid classes was quite consistent. The phospholipid primarily labeled was PC (81%), although other phospholipids were also labeled to lesser extents (phosphatidylethanolamine [PE], 11%; phosphatidylserine [PS]/PI, 6%; PA, 1.5%; and lyso-PC, 0.2% [Gruchalla *et al.*, 1990]). Figure 2 demonstrates that the presence of labeled PA increased dramatically at the earliest time examined (15 s) and peaked within 30 s of stimulation, coincident with the explosive exocytotic response of mast cells (Sullivan *et al.*, 1975; data

Figure 1. Direct and indirect pathways of 1,2-diacylglycerol generation.

not shown). Assuming that PC was the principal substrate for PA formation after stimulation of the high-affinity receptor for immunoglobulin E ($Fc\epsilon$ -RI), the increase in labeled PA represents 1–2% of the labeling initially present in PC before stimulation. Although these data are consistent with receptor-mediated activation of PLD, they are equally consistent with PLC-mediated formation of DAG followed by phosphorylation by DAG kinase. In fact, in early studies employing $^{32}P_i$ prelabeling, [^{32}P]PA was shown to begin to increase in $Fc\epsilon$ -RI stimulated mast cells within 10–15 s (Kennerly *et al.*, 1979a). Figure 2 demonstrates, however, that in the same experiments in which PA accumulation was examined, receptor-mediated accumulation of labeled DAG occurs somewhat less rapidly—a finding more consistent with PA acting as a substrate in the formation of DAG rather than the reverse. In addition to these data, activation of PLD in the mast cell is also supported by a dramatic increase in ethanol-dependent transphosphatidylolation as the result of $Fc\epsilon$ -RI stimulation (Gruchalla *et al.*, 1990),—findings similar to those of others in different model systems (Cabot *et al.*, 1988; Pai *et al.*, 1988; Lis-covitch and Amsterdam, 1989).

Rationale for examining cellular choline as a measure of PC-PLD activity

Whereas most recent studies focus on the formation of the lipid products of PLD-mediated hydrolysis or transphosphatidylolation (PA and phosphatidylethanol, respectively), the formation of choline as a result of PLD-catalyzed PC

hydrolysis represents a complementary approach to examining changes in PLD activity. Early studies in a variety of model systems examining the release of water-soluble products of cells prelabeled with [3H]choline primarily focused on the release of labeled choline, phosphorylcholine, and glycerophosphorylcholine into the extracellular environment—an approach making it difficult to assess the relative importance and location of PLD versus PLC in the formation of free choline and phosphorylcholine. Moreover, the ability of the labeled choline to be incorporated into a variety of compounds other than PC during long-term labeling seriously complicated interpretation of these findings inasmuch as careful characterization of the water-soluble labeled compounds was often not performed. Additionally, in terminally differentiated cells, the inability to label cellular PC to uniform specific activity with labeled choline suggested that the use of a mass-based assay of choline and phosphorylcholine would provide important information.

Receptor-dependent changes in PC-PLD activity as assessed by changes in the mass of intracellular choline

Figure 3 illustrates that the mass of intracellular choline increased dramatically from a basal level

Figure 2. IgE receptor-mediated changes in [3H]DAG and [3H]PA. After prelabeling purified mast cells with [3H]palmitic acid and extensive washing, we stimulated cells by IgE receptor crosslinking (Δ) or by medium alone ($\bullet$). After extraction, PA and DAG were separated from other phospholipids by TLC and labeling determined in the fluorographically identified bands by removing the labeled area and performing liquid scintillation spectrometry. Data points for PA are linked by solid lines, whereas those for DAG are linked by broken lines. Data represent mean $\pm$ SE of results obtained in three separate experiments, each performed in triplicate. Prelabeling (CPM/ 10^6 mast cells) before stimulation was PA, $11.6 \pm 2.5 \times 10^3$; DAG, $15.5 \pm 2.6 \times 10^3$; and PC, $8.7 \pm 2.2 \times 10^5$.

Figure 3. Intracellular choline mass during exocytosis. After purification, mast cells that were stimulated by IgE crosslinking (▲—▲) or remained unstimulated (●—●) were allowed to incubate at 37°C for the indicated times before stopping the reaction by the addition of cold medium and rapid separation of cells from medium using a microfuge. The mass of choline in the cell pellet was assayed by radioactive phosphorylation as described in detail in Methods. Data represent mean $\pm$ SE of data obtained in one of three similar experiments. The earliest time points are 20 s and 1 min after stimulation.

of 13.1 ± 1.2 pmol/10⁶ mast cells (mean $\pm$ SE) to levels that are 4- to 11-fold higher as a result of immunoglobulin E (IgE) receptor stimulation. The hypothesis that PLD is responsible for the increase in labeled PA accumulation predicts a coincident temporal association of the initial upslope of both labeled PA and choline mass because they are the two immediate products of PLD-mediated PC hydrolysis. As predicted, both the rise in choline mass (Figure 3) and the rise in labeled PA (Figure 2) peaked 20–30 s after stimulation. That the subsequent decline in choline mass and labeled PA occurs with different kinetic patterns does not undermine the hypothesis that PLD is of principal importance in the genesis of these intermediates, inasmuch as the subsequent metabolism of these two compounds occurs by vastly different pathways.

Of interest is not only the robust nature of the rise in mast cell choline mass but also its transience (Figure 3). Although previous data suggested that increased PLD-mediated transphosphatidylolation was greatest shortly after stimulation, considerable activity was still seen 5–10 min after Fc ϵ -RI stimulation (Gruchalla *et al.*, 1990). These data suggested that changes in intracellular choline mass were not exclusively the result of changes in PLD activity, but might also reflect changes in pathways that utilize intracellular choline. Although a concentration-dependent leakage of choline would be an ob-

vious explanation, this hypothesis was ruled out in experiments using brief prelabeling of intracellular pools of choline and phosphorylcholine by [³H]choline, which demonstrated that the rate of leakage of intracellular choline metabolites out of the cell is extremely limited and does not increase with stimulation (data not shown). As described in detail in a subsequent section, a more likely explanation is accelerated choline kinase-catalyzed formation of cellular phosphorylcholine, perhaps due to the combined effects of increased [choline]_{cytosol} and decreased [phosphorylcholine]_{cytosol} and/or active up-regulation of cytosolic choline kinase. On the basis of a uniform distribution within the cell and an average cell volume of 498 μm^3 for the mast cell (Kennerly, 1987b), the intracellular choline concentration can be estimated to be ~ 25 μM in unstimulated mast cells and 100–250 μM when choline levels rise 4- to 10-fold. That most mammalian choline kinases have K_m values for choline in the range of 50–250 μM and are inhibited by phosphorylcholine (reviewed by Ishidate, 1989) suggests that the increase in mast cell [choline]_{cytosol} coupled with a decrease in [phosphorylcholine]_{cytosol} (vide infra) may contribute to accelerated conversion of choline to phosphorylcholine.

Receptor-dependent changes in PC-PLC as assessed by changes in cell-associated phosphorylcholine

Because of the possibility that PC-PLC might be of principal importance in the formation of DAG in activated cells, the mass of phosphorylcholine was examined. In agreement with the findings of others in different tissues (Ishidate, 1989) the levels of phosphorylcholine were found to be ~ 15 -fold greater than those of choline (182 ± 19 pmol/10⁶ mast cells). Figure 4 demonstrates the surprising finding that Fc ϵ -RI stimulation caused a dramatic acceleration of the decline in phosphorylcholine levels in mast cells incubated in the absence of extracellular choline.

This unexpected finding raised several important issues. First, these data strongly suggest that, with the exception of the outer leaflet of the plasma membrane and the inner leaflet of the secretory granule (which would liberate phosphorylcholine to the extracellular environment), hydrolysis of PC by PLC was probably not increased as a result of Fc ϵ -RI stimulation (activation of PC-PLC would be expected to generate an increase in cellular phosphorylcholine). As mentioned above, the decline was not

Figure 4. Intracellular phosphorylcholine mass during exocytosis. In the same experiment that yielded Figure 3, the cell-associated phosphorylcholine mass was assessed in stimulated mast cells ($\blacktriangle$ — $\blacktriangle$) and compared with that observed in unstimulated cells ($\bullet$ — $\bullet$) using techniques described in detail in Methods.

due to significant loss of intracellular phosphorylcholine to the extracellular environment.

A possibility of great concern to us is that both the increase in intracellular choline and the fall in phosphorylcholine might have been due simply to an enhancement of receptor-mediated conversion of phosphorylcholine to choline. Indeed, the maximal net increase in the mass of choline and net decrease in phosphorylcholine are comparable (75–100 pmol). Although the finding that the nadir in phosphorylcholine did not kinetically coincide with the peak increase in choline mass undermined this explanation, a more careful assessment of this hypothesis was felt to be essential and is addressed in the next section.

Receptor-dependent changes in the metabolism of intracellular choline metabolites

Figure 5 illustrates the pattern of labeling of choline-containing compounds during a brief incubation of mast cells with [3 H]choline. That labeling of phosphorylcholine was found to be 7- to 15-fold that of choline with the chosen conditions is in accord with the relative mass of these metabolites in unstimulated cells (14-fold). After prelabeling, a nearly instantaneous reduction of the specific activity of extracellular choline to 10% of its original value (without any change in the concentration of choline) was accomplished, and a chase experiment was performed to examine the fate of labeled choline-containing products in the absence and presence of Fc ϵ -RI stimulation. Prelabeling

conditions were chosen so that both 1) the choline and phosphorylcholine pools were labeled nearly to isotopic equilibrium (>80% for choline, >60% for phosphorylcholine; data not shown) and 2) PC labeling was modest (<10% of final specific activity). These conditions were chosen so that the 1–2% hydrolysis of [3 H]PC to choline caused by Fc ϵ -RI stimulation would have a minimal impact on the presence of [3 H]choline. The purpose of this experiment was to eliminate the hypothesis that the dramatic receptor-dependent rise in choline mass (Figure 3) took place not as the result of PC hydrolysis by PLD, but rather by the simple conversion of phosphorylcholine to choline. This hypothesis predicts that, during the chase period, receptor stimulation would cause both 1) an acceleration of the decline in labeled phosphorylcholine and 2) a dramatic increase in labeled choline (because the specific activities of phosphorylcholine and choline are similar and the mass of the choline pool is so much smaller than that of phosphorylcholine). However, Figure 6 demonstrates clearly that Fc ϵ -RI stimulation caused only modest changes in the levels of labeled intracellular choline but dramatic acceleration of the decrease in labeling of phosphorylcholine—findings that rule out the possibility that phosphorylcholine hydrolysis accounts for the findings in Figures 3 and 4. That a modest receptor-dependent increase in choline labeling (<50%) is seen (at the 1-min time point in 2 of the 4 experiments) is consistent with isotopic dilution of choline by the dramatic liberation of low spe-

Figure 5. Prelabeling cellular choline metabolites with [3 H]choline. By the use of conditions described in Methods, mast cells were pre-labeled with [3 H]choline for the indicated times before chilling, washing, extracting cellular choline metabolites, and separating them by TLC. Data represent means $\pm$ SE of triplicate values obtained in one of four similar experiments.

Figure 6. Receptor-dependent changes in labeled choline metabolites. Cells were prelabeled with [³H]choline for 8 min as detailed in Methods and illustrated in Figure 5. After prelabeling, the isotopic specific activity was reduced by 90% by increasing the total volume 10-fold with buffer containing unlabeled choline at the same mass concentration (2.5 μ M). Exactly 2 min later, cells were stimulated by Fc ϵ -RI crosslinking and reactions halted at the indicated times by adding cold buffer. After rapid sedimentation of cells, the cell-associated, water-soluble choline metabolites were extracted, separated by TLC, and assessed for radioactivity by liquid scintillation spectrometry. Data represent means of triplicate observations in one of four similar experiments. SE bars are omitted for the sake of clarity, but in no case exceeded 10% of the mean value. Statistically significant changes induced by stimulation ($p < 0.05$) occurred in all metabolites at the 1-, 3-, and 10-min time points, except for choline, which was significant only at the 1-min time point.

cific activity choline from PC into the choline pool 20 s after stimulation, which then slowed the rate of [³H]choline disappearance—despite the likely receptor-dependent acceleration of the flux of choline mass through the pathway of PC biosynthesis (choline $\rightarrow$ phosphorylcholine $\rightarrow$ CDP-choline $\rightarrow$ PC) as described below.

A more attractive hypothesis is that the decline in phosphorylcholine reflects accelerated conversion of phosphorylcholine to CDP-choline for the resynthesis of PC lost, perhaps, as the result of PLD-mediated formation of PA. Indeed, Figure 6 is consistent with this hypothesis in that the declines in [³H]CDP-choline and [³H]phosphorylcholine during this chase period closely matched the increase in [³H]PC. In earlier studies from our laboratory (Kennerly *et al.*, 1979a) we demonstrated that, when CDP-choline becomes labeled by incubation of cells with ³²P_i, the rate of [³²P]PC synthesis increased dramatically as the result of Fc ϵ -RI stimulation.

Changes in extracellular choline and phosphorylcholine in Fc ϵ -RI-stimulated mast cells

In an effort to assess the presence of cellular PLC and PLD activities toward PC, a number of

studies have examined the release of labeled choline and its metabolites into the medium from cells prelabeled with [³H]choline in response to either relevant receptor stimulation or activation of PKC by phorbol esters (Guy and Murray, 1982; Besterman *et al.*, 1986; Daniel *et al.*, 1986; Bocckino *et al.*, 1987; Irving and Exton, 1987; Takuwa *et al.*, 1987; Cabot *et al.*, 1988; Pai *et al.*, 1988; Slivka *et al.*, 1988; Agwu *et al.*, 1989; Billah *et al.*, 1989; Liscovitch and Amsterdam, 1989; Martinson *et al.*, 1989; Pessin *et al.*, 1990). Because many of these studies did not separate and identify the labeled water-soluble compounds, and none addressed the mass of choline metabolites, we sought to apply a mass-based analysis in the assessment of potential changes in extracellular choline and phosphorylcholine as the result of Fc ϵ -RI stimulation in mast cells. Figures 7 and 8 demonstrate that the mass of both choline and phosphorylcholine in the extracellular medium increase significantly with physiological stimulation.

The increase in extracellular phosphorylcholine (from a basal level of 10–15 to 45–55 pmol/10⁶ cells) with stimulation (Figure 7) was unexpected in the face of a decline in intracellular phosphorylcholine mass. Because receptor-dependent leakage of intracellular choline and its metabolites was not observed (data not shown), the increase in extracellular phosphorylcholine suggested the provocative hypothesis that receptor-dependent, PLC-mediated hydrolysis of PC might occur in the outer leaflet of the plasma membrane or the inner leaflet of the secretory granule membrane (PLC-mediated hydrolysis of PC at the latter site would result in increases in

Figure 7. Extracellular phosphorylcholine mass during exocytosis. In the same experiment that yielded Figures 3 and 4, a sample of the cell-free supernatant of stimulated mast cells (▲—▲) or unstimulated cells (●—●) was examined for the presence of choline.

Figure 8. Extracellular choline mass during exocytosis. In the same experiment that yielded Figures 3, 4, and 7, a sample of the cell-free supernatant of stimulated mast cells (▲—▲) or unstimulated cells (●—●) was examined for the presence of phosphorylcholine.

extracellular phosphorylcholine as the result of exteriorization of this membrane surface during exocytosis). Purified secretory granules obtained from unstimulated cells were shown to be nearly devoid of choline and phosphorylcholine, making it unlikely that the increase in extracellular phosphorylcholine was simply due to exocytosis of preformed choline or phosphorylcholine during $Fc\epsilon$ -RI-initiated secretion (data not shown).

More quantitatively impressive was the increase in extracellular choline mass occurring with $Fc\epsilon$ -RI stimulation (Figure 8). The rise in extracellular choline differed from that of intracellular choline (Figure 3) in being slower to develop (peaking 1–3 min after stimulation). As mentioned earlier, no experimental support for receptor-dependent accelerated leakage of intracellular choline was obtained, although the slow increase in extracellular choline seen both in unstimulated and stimulated cells from 3 to 10 min was consistent with the observed modest rate of leakage of [3 H]choline (data not shown). As described above for the accumulation of extracellular phosphorylcholine, the data related to extracellular choline accumulation suggested that PC hydrolysis by PLD might be occurring either in the outer leaflet of the plasma membrane or the inner leaflet of the granule membrane. Studies by Hattori and Kanfer (1985) demonstrated that PLD activity was found in synaptosomes—a finding reinforcing the hypothesis that secretory granule membranes may be important sites of PLD activation.

Although the PC-PLC-mediated increase in extracellular phosphorylcholine was modest (net increase of 30–40 pmol/10⁶ cells), PLD ac-

tivity at this same site (net increase in choline of 175–225 pmol/10⁶ cells) significantly exceeded the increase seen in “intracellular sites” (80–140 pmol/10⁶ cells). Although it is tempting to propose that more vigorous PC-PLD activity exists in subcellular sites giving rise to extracellular choline, dramatically different capacities for removal exist in the cytosol compared with the extracellular medium. Thus, the receptor-dependent increase in intracellular choline mass (80–140 pmol/10⁶ mast cells) coupled with our observation of increased PC resynthesis (Figure 6; choline → phosphorylcholine → CDP-choline → PC) suggests that this figure may considerably underestimate PLD activity, because cytosolic choline removal is probably accelerated as the result of stimulation.

Concluding comments

These studies provide additional support for the emerging importance of receptor-dependent increases in PLD-catalyzed PC hydrolysis as an element in signal transduction. Although other studies have demonstrated receptor-mediated changes in choline mass in hepatocyte plasma membranes (Bocckino *et al.*, 1987) and preliminarily in neutrophils (Truett *et al.*, 1989), these studies are the first that provide a detailed mass-based assessment of the activity of both PLC and PLD toward PC.

The molecular species similarity of cellular PC and the incremental mass of DAG seen with $Fc\epsilon$ -RI stimulation strongly suggested that pathways capable of converting PC to DAG required careful examination (Kennerly, 1987a, 1990; Wright *et al.*, 1988; Pessin *et al.*, 1990). Although our previous studies in this model system (Gruchalla *et al.*, 1990) and an increasing number of others indicated that PLD activity is increased during physiologically relevant stimulation, the quantitative importance of either the direct or indirect pathways of DAG formation from PC (Figure 1) needed more careful assessment. Because leakage of intracellular choline out of the mast cell appears not to have been quantitatively important, it seems reasonable to estimate that the total receptor-dependent PC-PLD activity is ≥ 250 –350 pmol/10⁶ cells (the sum of the increases in the mass of choline from both intracellular and extracellular compartments). This observation is in gratifyingly good agreement with less quantitatively meaningful estimates developed by experiments in mast cells in which PC was prelabeled with [3 H]palmitic acid (Gruchalla *et al.*, 1990), where the accumulation of

labeled PA (or phosphatidylethanol in the presence of exogenous ethanol) after Fc ϵ -RI stimulation represented $\sim 1.5\%$ of labeled PC (Gruchalla *et al.*, 1990). Given that the mass of PC is 17.6 nmol PC/ 10^6 mast cells (Kennerly, 1990), our currently observed production of ≥ 250 pmol choline/ 10^6 cells suggests that receptor stimulation causes PLD hydrolysis of $\geq 1.4\%$ of cellular PC.

Although the PA generated by PLD activation may have a variety of metabolic fates, its conversion to DAG by PA-PHase by the indirect pathway (Figure 1) may represent an important mechanism of DAG generation during Fc ϵ -RI stimulation in mast cells. Indeed, the two estimates of total PC-PLD activity compare favorably with existing data with regard to 1) comparable magnitudes of the increase in DAG mass (100–250 pmol/ 10^6 mast cells [Kennerly *et al.*, 1979b; Kennerly, 1987a, 1990]) compared with that of choline mass (250–350 pmol/ 10^6 mast cells; Figures 3 and 8) and 2) the kinetic delay of the increase in DAG mass compared with that of choline (DAG levels begin to increase 1 min after stimulation, are 50% of maximal at 3 min, and peak at 10 min [Kennerly *et al.*, 1979b; Kennerly, 1990]) that would be expected if PLD-mediated PA formation (and choline liberation) preceded the conversion of PA to DAG rather than PA being derived from DAG by phosphorylation. To our surprise, in unpublished work from our laboratory, we have observed that $\sim 50\%$ of the cellular PA-PHase activity in mast cells is associated with highly purified secretory granules (Duffy and Kennerly, as-yet-unpublished observations)—an observation underscoring the importance of examining the secretory granule membrane as a site of receptor-initiated phospholipid metabolism.

Further, the quantitatively relatively modest Fc ϵ -RI-mediated increase in the mass of extracellular phosphorylcholine, coupled with the concomitant decrease in intracellular phosphorylcholine, argues against a major role for PC-PLC during receptor-dependent mast cell activation. Only modestly limiting the certainty of this conclusion is our observation that mast cell stimulation was associated with enhanced resynthesis of PC—an observation that might tend to antagonize the accumulation of intracellular phosphorylcholine caused by an increase in the activity of PC-PLC.

Although experimental evidence directly examining the activity of PLD toward phospholipid substrates other than PC is not addressed by the current studies, our molecular species analyses of DAG and PC (Kennerly, 1990) suggest

that PC is much more likely to be the principal substrate in the receptor-dependent formation of cellular DAG than is PI, PE, or PS (Kennerly, 1987a; Wright *et al.*, 1988; Kennerly, 1990; Pessin, 1990). However, PC may not be the principal substrate in the formation of cellular PA, although preliminary molecular species analysis of mast cell PA indicates close similarity to PC (unpublished observations). Supporting the importance of PC as a substrate for cellular PLD are recent studies by Pessin *et al.* (1990) in fibroblasts demonstrating that labeled choline, but not serine or ethanolamine, is released from receptor-stimulated cells in which phospholipids had been prelabeled by incubation with either labeled choline, serine, or ethanolamine.

The finding that the outer leaflet of the plasma membrane and/or the inner leaflet of the secretory granule may be important sites of PC hydrolysis (both by PLC and PLD) strongly encourage careful subcellular studies of these receptor-dependent activities. Although the inner leaflet of the plasma membrane has received primary attention as a site of receptor-dependent phospholipase activation, the ability to generate lipid second messengers and/or lipid molecules capable of facilitating membrane fusion in the secretory granule membrane may be of great importance to the process of exocytosis.

Materials and methods

Reagents

Materials and their sources are as follows: unless otherwise indicated, all salts, small molecules, and commercially prepared proteins and enzymes, Sigma Chemical (St. Louis, MO); organic solvents, J.T. Baker purchased from Fisher Scientific (Houston, TX); male Sprague Dawley rats, Southwest Breeding Laboratories (Midlothian, TX) and Sasco (Omaha, NE).

Isolation and purification of mast cells

Mast cells were obtained by lavage of the peritoneal and pleural cavities. After saturation of Fc ϵ -RI with a monoclonal IgE anti-DNP, cells were purified to $>98\%$ homogeneity by discontinuous Percoll density gradients as described in detail elsewhere (Gruchalla *et al.*, 1990).

Receptor stimulation of mast cells

Unlabeled mast cells ($0.8\text{--}1.2 \times 10^6$ in experiments assessing choline mass) and [^3H]palmitic acid-labeled mast cells ($2.5\text{--}3.5 \times 10^6$ cells) were similarly brought to 37°C for 10 min before stimulation. To examine physiologically relevant IgE receptor stimulation, we exposed cells to DNP $_{1-7}$ -human serum albumin (100 ng/ml) in the presence of sonically dispersed PS (15 $\mu\text{g/ml}$; a necessary cofactor of Fc ϵ -RI-mediated mast cell activation [Goth *et al.*, 1971]) and compared with cells exposed to PS alone (total volume, 250 μl). Secretion began within 15–30 s, and maximal exocytosis (25–

60% release of the secretory granule marker β -hexosaminidase) was observed within 5 min of crosslinking Fc ϵ -RI (Sullivan *et al.*, 1975; data not shown).

Conceptual overview

We have described the extraction and quantitation of cell-associated choline and phosphorylcholine in a previous publication (Murray *et al.*, 1990). This procedure is based on the selective ability of choline (but not phosphorylcholine) to form a heptanone-soluble ion pair with the tetraphenylborate ion. Cellular choline (or choline derived from cellular phosphorylcholine by alkaline phosphatase-catalyzed hydrolysis) was quantitated by choline kinase-mediated formation of [32 P]phosphorylcholine in linear proportion to the amount of choline present over a 3 log range.

Extraction of cellular choline

As described in detail elsewhere (Murray *et al.*, 1990), cell pellets were extracted employing a modification of the method of Bligh and Dyer (1959). After addition of [3 H]choline to assess recovery of cellular choline and yield during its quantitation, the extract was centrifuged to remove insoluble material, and phase separation of the supernatant was accomplished by adding chloroform and water. Choline was selectively extracted from a sample of the upper methanol/water phase using the ion pair reagent sodium tetraphenylboron (TPB) in 4-heptanone. Free choline in aqueous solution was obtained by extracting an aliquot of the TPB/choline complex in heptanone with 0.1 N aqueous HCl. After brief centrifugation, the heptanone layer was removed by vacuum aspiration, and a portion of the choline containing aqueous phase was removed and dried under N $_2$ at 40°C.

Extraction of cellular phosphorylcholine

Cells were treated exactly as described above except that the Bligh and Dyer upper phase was doubly extracted with TPB/heptanone to remove >95% of the cell-derived choline (Murray *et al.*, 1990). The choline-depleted, phosphorylcholine-containing water/methanol was washed once with 1 ml of heptanone (to remove remaining TPB) and dried.

Conversion of phosphorylcholine to choline

After removing choline from the cell extracts, we solubilized cellular phosphorylcholine (present in the dried phosphorylcholine-containing extract described in the previous section) and converted it to choline *in vitro* by alkaline phosphatase-catalyzed hydrolysis (described in detail elsewhere [Murray *et al.*, 1990]).

Extraction of choline and phosphorylcholine released from cells into the incubation medium

After sedimenting cells suspended in 0.5 ml of buffer, we examined 0.4 ml of the supernatant for the presence of choline by diluting with 1.1 ml of distilled water followed by extraction using 1 ml of TPB heptanone as described above. As in the analysis of cellular phosphorylcholine, after removing >95% of the choline by twice extracting the cell-free supernatant with TPB heptanone, we converted the remaining phosphorylcholine to choline using alkaline phosphatase followed by extraction of the choline product using TPB heptanone.

Quantitative analysis of cell-derived choline

Using methods detailed elsewhere (Murray *et al.*, 1990), we determined the mass of choline by its radioactive phosphorylation using γ [32 P]ATP (ICN, Irvine, CA) and commercially prepared choline kinase. The enzymatically generated [32 P]phosphorylcholine product was separated from labeled ATP by both precipitation of ATP with barium acetate and anion exchange chromatography on minicolumns containing Dowex AG-1-X8 and quantitated by liquid scintillation spectrometry. Aside from the content of cellular choline, no interference by the cellular extract was observed on exogenously added choline at the cell numbers utilized in these experiments (data not shown).

Prelabeling mast cell lipids with [3 H]palmitic acid

In some experiments 10–20 million purified mast cell lipids were prelabeled by exposure to 200 μ Ci of the labeled fatty acid, [3 H]palmitic acid (30 Ci/mmol; New England Nuclear, Boston, MA) in a total volume of 1–2 ml of buffer containing 0.1 mg/ml lipid poor bovine serum albumin (BSA; Calbiochem, La Jolla, CA) for 1 h at 37°C. Cells were sedimented and washed twice in lipid poor BSA-containing buffer (Gruchalla *et al.*, 1990), incubated for 15 min at 37°C, and washed once more before stimulation. Cellular lipids were extracted with the previously described Bligh and Dyer extraction, and labeled PA and DAG were separately isolated by TLC using a double one-dimensional solvent system (Kennerly, 1987a) and hexane:diethyl ether:acetic acid (60:40:1 by volume), respectively.

Studies utilizing [3 H]choline incorporation

In experiments assessing potential leak of intracellular choline during mast cell stimulation, 5–8 $\times 10^6$ cells labeled with 100 μ Ci of ([3 H]methyl)choline for 15 min were chilled, washed twice with buffer not containing labeled choline, and aliquoted (2.5–3.5 $\times 10^6$ cells) in 100 μ l to each tube. After being rewarmed to 37°C for 2 min, cells were stimulated by Fc ϵ -RI crosslinking and, at the times of interest, processes were halted by addition of cold buffer followed by rapid sedimentation of cells in a microfuge. Labeled choline and its metabolites associated with both the cell pellet and the supernatant were assessed. The cell pellet was subjected to Bligh and Dyer extraction and the water-soluble choline metabolites present in the upper phase were separated by TLC (*vide infra*), whereas labeled PC in the lower chloroform phase was isolated by TLC (chloroform:methanol:28% NH $_4$ OH [65:35:5 by volume] on silica gel G). Labeled products were visualized by fluorography using EnHance (New England Nuclear) and quantitated by liquid scintillation spectrometry. Data are corrected for incomplete recovery by parallel extraction, TLC, and counting of authentic [3 H]choline and [3 H]phosphorylcholine. For water-soluble [3 H]choline outside the cell, aliquots of the supernatant obtained after cellular sedimentation were both directly assessed for total labeled choline products and subjected to ion pair extraction using TPB as described above, extracted into acid, dried, and quantitated by liquid scintillation spectrometry to distinguish labeled choline from other metabolites.

In pulse-chase experiments examining the fate of intracellular choline and its metabolites, 2.5–3.5 $\times 10^6$ mast cells were prelabeled with 4 μ Ci of [3 H]choline for exactly 8 min in a final concentration of choline of 2.5 μ M in 50 μ l. Cells were diluted to a final volume of 500 μ l with prewarmed buffer containing unlabeled choline at exactly the same concentration (2.5 μ M) to acutely reduce the specific activity

of extracellular choline by 90%. Exactly 2 min later, cells were stimulated by Fc ϵ -RI crosslinking and, at the indicated times, cellular metabolic processes were halted by adding 2 ml of ice-cold buffer and cells sedimented by centrifugation and washed with buffer devoid of labeled choline. Labeled choline and its water-soluble metabolites in the washed cell pellet were obtained in the water/methanol phase of a Bligh and Dyer extract, separated by TLC (vide infra), localized by fluorography, and quantitated by liquid scintillation spectrometry.

Separation of choline, phosphorylcholine, and CDP-choline by TLC

The method of Vance *et al.* (1980) was used exactly as described. Briefly, samples were applied in methanol:water (1:1) 2 cm from the bottom of a 20 $\times$ 20-cm Sil G plastic-backed TLC plate (Brinkmann, Westbury, NY). After sample zones were completely dry, development was accomplished in methanol:0.5% NaCl:28% NH₄OH (50:50:1 by volume) to a height of 12 cm. Labeled bands were visualized by fluorography after spraying with EnHance and identified by comigration with unlabeled standards run in parallel lanes. Labeled areas were scraped from the plate, and radioactivity was assessed by liquid scintillation spectrometry.

Analysis of data

Unless otherwise indicated in figure legends, data represent the means $\pm$ SE of triplicate values obtained from one of two to four experiments. The absence of error bars indicates a SE smaller than the size of the symbol.

Acknowledgments

The authors wish to thank Malia Newell for her expert assistance in the preparation of this manuscript. This work was supported by grants from the National Institutes of Health (RO1-AI-22277) and the Burroughs Wellcome Foundation/American Academy of Allergy and Immunology (Developing Investigator Award in Immunopharmacology of Allergic Disease).

Received: November 26, 1990.

Revised and accepted: February 12, 1991.

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