Thapsigargin-Induced Secretion Is Dependent on Activation of a Cholera Toxin-Sensitive and Phosphatidylinositol-3-kinase-Regulated Phospholipase D in a Mast Cell Line

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ABSTRACT
Release of secretory granules by rat RBL-2H3 mast cells is mediated primarily through activation of protein kinase C (PKC) and elevation of cytosolic free calcium ([Ca\(^{++}\)]\(_i\)). Here, we show that secretion was also dependent on the activation of a cholera toxin-sensitive phospholipase (PL) D in cells stimulated with thapsigargin. Wortmannin, LY294002, butanol, propranolol and PLD inhibitor Ro31-7549 inhibited responses to variety of secretagogues in a manner consistent with the notion that secretion was regulated by both PLD and PKC in a phosphatidylinositol-3-kinase-dependent manner. The effects of these inhibitors, however, were especially pronounced in cells activated by thapsigargin. This stimulant induced minimal stimulation of PLC but measurable activation of PLD, as assessed by formation of phosphatidylethanol in the presence of ethanol. The activation of PLD was suppressed by inhibitors of phosphatidylinositol-3-kinase and was dependent on a rise in [Ca\(^{++}\)]\(_i\), because thapsigargin failed to activate PLD and secretion when elevation of [Ca\(^{++}\)]\(_i\) was blocked. Treatment of cells with cholera toxin resulted in selective and similar enhancements in the activation of PLD and secretion by thapsigargin, whereas stimulation of PLC and PLA\(_2\) was unaffected. A role for PKC was indicated by the blockade of secretory response to thapsigargin by the PKC inhibitor Ro31-7549 and by the ability of the PKC agonist phorbol-12-myristate-13-acetate to reverse the inhibition of secretion by inhibitors of PLD. Such results suggested that thapsigargin, by causing substantial increases in [Ca\(^{++}\)]\(_i\), induced secondary signals via PLD and PKC that synergized a calcium signal for secretion.

The rat mast cell line (RBL-2H3) can be stimulated to secrete intracellular granules through multivalent binding of antigen to receptor-bound IgE. The resultant aggregation of the receptors (of the FcεRI category) initiates a cascade of events that include the activation of the cytosolic tyrosine kinases Lyn and Syk, (reviewed in Beaven and Baumgartner, 1996) and the tyrosine phosphorylation of various proteins (Benhamou and Siraganian, 1992; Benhamou et al., 1992), among them PLCγ (Park et al., 1991). The activation of PLC (Park et al., 1991) as well as PLD (Gruchalla et al., 1990; Lin et al., 1991a, 1994), along with sustained elevation of diglycerides (Lin et al., 1991a) and mobilization of Ca\(^{++}\) from intracellular and extracellular sources (Cho et al., 1993; Millard et al., 1989), results in the activation of serine/threonine kinases. These include PKC (White and Metzger, 1988; Ozawa et al., 1993), and Ca\(^{++}\)/calmodulin-activated myosin light-chain kinase (Choi et al., 1994; Teshima et al., 1989).

Secretion also is induced by carbachol in a mutated cell (RBL-2H3-m1) line that expresses muscarinic m1 receptors (Choi et al., 1993). Carbachol elicits similar responses to antigen (Choi et al., 1993) except they are not dependent on Syk (Hirasawa et al., 1995) but instead on the activation of PLCβ via the G protein G\(_{q/11}\).2 Reconstitution studies with washed permeabilized cells, which lose all isozymes of PKC, indicate that activation of PKC and a modest elevation of [Ca\(^{++}\)]\(_i\) provide the necessary and sufficient signals for secretion (Ozawa et al., 1993).

RBL-2H3 cells also secrete in response to pharmacological stimulants that directly mobilize calcium. These stimulants include the Ca\(^{++}\) ionophores ionomycin and A23187 (Lo et al., 1987) and thapsigargin (Smith et al., 1991; Ali et al., 1994), which elevates [Ca\(^{++}\)]\(_i\) by blocking uptake of Ca\(^{++}\) into inositol-1,4,5-trisphosphate-sensitive stores (Putney and Bird, 1993). Low concentrations of these reagents induce physiologically significant increases in [Ca\(^{++}\)]\(_i\) but not secre-
tion (Lo et al., 1987; Ali et al., 1994), unless given in combination with PMA to promote the necessary activation of PKC in RBL-2H3 cells (Choi et al., 1994; Lo et al., 1987). These agents, however, can induce secretion at concentrations that result in substantial stimulation of PLD (Nakashima et al., 1991; Lin and Gilfillan, 1992) and PKC (Choi et al., 1994) but cause minimal stimulation of PLC (Lo et al., 1987; Ali et al., 1994). The physiological activator of PKC, the diglycerides, can be generated through the actions of PLC and PLD, the latter in conjunction with phosphatidate phosphohydrolase (Nishizuka, 1995). It has not been established, however, whether activation of PLD alone is sufficient for mediating PKC-dependent secretion.

Studies of the physiological role of PLD activation have been hampered by a lack of specific inhibitors of PLD and phosphatidate phosphohydrolase, which rapidly converts the PLD product, phosphatidic acid, to diglyceride (Billah and Anthes, 1990; Exton, 1990). Wortmannin is reported to inhibit PLD (Reinhold et al., 1990; Bonser et al., 1991), but this compound also inhibits activation of PI 3-kinase (Yano et al., 1993) and one form of PI 4-kinase (Downing et al., 1996) at nanomolar concentrations. Butanol, an inhibitor of PLD (Yang et al., 1967), and propanolol, an inhibitor of phosphatidate phosphohydrolase (Koul and Hauser, 1987; Jamal et al., 1991; Lin et al., 1991a), both inhibit PKC activity over the same range of doses (Sozzani et al., 1992; Slater et al., 1993). Activation of PLD in antigen and ionomycin-stimulated RBL-2H3 cells (Ali et al., 1996) is thought to be associated with secretion on the basis that propranolol inhibits PLD and secretion in these cells (Lin et al., 1991a, 1991b). These studies failed, however, to consider the possible inhibition of PKC. Thus, although it widely assumed that PLD-derived products provide a significant source of diglycerides for activation of PKC and for mediation of PKC-dependent responses such as secretion, no unambiguous evidence exists for this hypothesis.

In the present study, we reexamined the effects of the inhibitors of the PLD pathway on responses to the various secretagogues in RBL-2H3 cells. Of these secretagogues, thapsigargin-induced secretion was most readily suppressed by all inhibitors, and additional experiments were designed to establish that activation of PLD was essential for this secretion. Advantage was taken of our recent finding3 that treatment with cholela toxin enhanced activation of PLD by various stimulants via a pathway that was distinct from the stimulation of PLD by GTPyS and small monomeric GTP-binding proteins (see Frohman and Morris, 1996; Exton, 1997). These experiments demonstrated concordant enhancement of PLD activation and secretion in toxin-treated cells, and they further indicated that both responses were equally suppressed by inhibitors of PI 3-kinase. Other experiments in thapsigargin-stimulated cells also suggested that PLD-derived products acted primarily through calcium-independent isoform or isoforms of PKC.

Materials and Methods

Reagents. Wortmannin, thapsigargin, PMA and Ro31-7549 were from LC Laboratories (Woburn, MA). LY294002 was from BIOMOL (Plymouth Meeting, PA). Calcium ionophore A23187 and G6-6976 were from Calbiochem (La Jolla, CA). p-Nitrophenyl-N-acetyl-ß-D-glucosamide and phosphatidic acid were from Sigma Chemical (St. Louis, MO). Propranolol was from Ayerst Laboratories (New York, NY). Carbachol was from Aldrich Chemical (Milwaukee, WI). Butanol was from Mallinkrodt (St. Louis, MO). Phosphatidylethanol was from Avanti Polar Lipids (Pelham, AL). Bacterial toxins were from List Biologicals (Campbell, CA). Streptolysin-O was from Murex (Dartford, UK). Radiolabeled compounds were from DuPont-New England Nuclear (Boston, MA). The antigen, DNP-BSA and DNP-specific monoclonal IgE were gifts from Dr. Henry Metzger (National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health).

Cell culture. Experiments were performed with RBL-2H3 cells that had been transfected with the gene for muscarinic m1 receptors (Choi et al., 1993). RBL-2H3 cells were maintained as monolayer cultures and harvested by trypsinization as described previously (Ali et al., 1990). Cells were then transferred to 24-well (2 × 10⁵ cells/ml/well) or 6-well (2 × 10⁶ cells/ml/well) cluster plates in modified Eagle’s medium with Earle’s salts, supplemented with 15% fetal bovine serum. Cultures were incubated overnight in complete growth medium at 37°C with 0.5 µg/ml DNP-IgE to yield 100% occupancy of FcεRI by IgE (Yamada et al., 1992).

Stimulation of cells and other experimental conditions. For each experiment, the cells were washed twice with the glucose-saline, PIPES-buffered medium (Ali et al., 1994) before the addition of the same buffer (0.2 ml/well for 24-well plates and 1.0 ml/well for 6-well plates). The cultures were preincubated at 37°C with the appropriate concentration of wortmannin or Ro31-7549 for 10 min or with propranolol or butanol for 5 min before the addition of the stimulants. PMA was added with these inhibitors as indicated. Vehicle was added to cultures where appropriate as a control. Except where noted otherwise, stimulants were added at concentrations that were optimal for secretion. Reactions were terminated after a 15-min stimulation by placing the cultures on ice. Medium was removed and cells were lysed in 0.5 ml (24-well plate) or 2 ml (6-well plate) 0.1% Triton X-100 unless otherwise noted.

Other experimental conditions included depolarization of cells by substituting K⁺ for Na⁺ in the external medium to block entry of external calcium and elevation of [Ca²⁺]i (Ali et al., 1994), treatment with 1 µg/ml cholera toxin for 4 hr in complete growth medium at 37°C (Ali et al., 1990) and the permeabilization of cells with streptolysin-O (Ozawa et al., 1993). For the latter procedure, however, cells were incubated with 0.7 unit of streptolysin-O for 1 min to minimize loss of PKC isozymes, as determined by immunoblotting and secretory response to antigen (Ozawa et al., 1993). Otherwise, all procedures performed exactly as described in the cited references.

Stock solutions of A23187, thapsigargin, PMA, wortmannin, LY294002, G6-6976 and Ro31-7549 were prepared in dimethylsulfoxide. They were diluted directly into the PIPES-buffered medium to give the required final concentration of drug and a concentration of dimethylsulfoxide of ≤0.1% (v/v). All other reagents were dissolved in the medium.

Measurement of release of hexosaminidase, [H]inositol phosphates and [¹⁴C]arachidonic acid. Secretion was determined by measurement of the release of hexosaminidase, a granule marker, by the hydrolysis of p-nitrophenyl-N-acetyl-ß-D-glucosamido to the chromophore, p-nitrophenol, as described elsewhere (Ozawa et al., 1993). Absorbance (410 nm) was measured in a microtitrator plate reader. For measurement of generation of radiolabeled metabolites, cells were incubated overnight with myo-[¹⁴C]inositol (4 µCi/ml) and [¹⁴C]arachidonic acid (1 µCi/ml) in complete growth medium. Generation of radiolabeled inositol phosphates and arachidonic acid was determined as described previously (Maeyama et al., 1986; Yamada et al., 1992).

After stimulation, samples of medium and cell lysates were assayed to determine the percentage of hexosaminidase and [¹⁴C]arachidonic acid that was released into the medium exactly as de-
Further incubation of the cultures in the presence of 2 specific IgE in six-well plates as described above and then labeled by dylethanol. RBL-2H3 cells were incubated overnight with DNP-absence of stimulant (hexosaminidase, where noted, values were corrected for spontaneous release in the lysis of [3H]inositol phospholipids (Maeyama phospholipids in unstimulated cultures to give a measure of hydrophosphates formed was calculated as a percentage of [3H]inositol [3H]myristic acid for 90 min. Cultures were washed with the glucose-saline, PIPES-buffered medium as described in the previous section. Cultures were then incubated in the presence of 0.3% ethanol for 10 min before stimulation. Under these conditions, phosphatidylethanol is formed by a transphosphatidylation reaction. Unlike phosphatidic acid, phosphatidylethanol is a PLD-specific product and accumulates within the cell because of its slow metabolism (Dennis et al., 1991).

Radiolabeled phosphatidic acid and phosphatidylethanol were isolated and assayed by minor modifications of previously described procedures (Bligh and Dyer, 1959; Kennerly, 1987). At the indicated times, the reaction was terminated by the addition of 3.75 ml of a mixture of chloroform-methanol 4 N HCl (100:200:2, v/v/v) to form a monophasic mixture. The resultant monophasic mixture was separated into two phases by the addition of 1.25 ml of chloroform, which contained 30 μg of unlabeled phosphatidic acid and phosphatidylethanol as carriers, and 1.25 ml of 0.1 N HCl. Of the lower chloroform phase, 2.0 ml was evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of a mixture of chloroform-methanol (2:1). A 25 μl sample of this solution was spotted onto a thin-layer silica gel plated sheet that was then subjected to thin-layer chromatography (Tomhave et al., 1994). With this procedure, a mixture of chloroform-methanol-glacial acetic acid (65:15:2, v/v/v) was used for development of the chromatogram. The sheet was air-dried and then exposed to iodine vapor to visualize the phospholipids. Phosphatidic acid and phosphatidylethanol were then cut from the sheets for assay of tritium. The amounts of radiolabeled phospholipids were calculated as a percentage of total radiolabel that accumulated in cells (Dennis et al., 1991), including RBL-2H3 cells (data not shown).

In the above experiments, the decreases in secretion and accumulation of [3H]phosphatidylethanol were highly correlated (P < .001). In addition, the inhibition of secretion was dependent on the concentration of wortmannin, although the secretagogues were affected differently; thapsigargin-induced secretion was the most affected, whereas carbachol and A23187-induced secretion were the least affected (fig. 1C).
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Concentrations of wortmannin required for 50% inhibition (IC50) of the thapsigargin-, antigen-, carbachol- and A23187-induced secretion were, respectively; 23 ± 3, 41 ± 6, 71 ± 19 and ≥100 nM (mean ± S.E.M. for 6 experiments). Wortmannin, however, inhibited activation of PLC, possibly as a consequence of inhibition of PI 4-kinase (Downing et al., 1996), as well as PLD. The generation of [3H]inositol phosphates and secretion in antigen- or carbachol-stimulated cells were inhibited equally and in a highly correlated manner (correlation P < .001 with both stimulants for experiments shown in fig. 2A; data not shown). Similar correlations were difficult to determine in the thapsigargin- or A23187-stimulated cells because, as in previous studies, these two secretagogues minimally stimulated inositol phospholipid hydrolysis (Lo et al., 1987; Ali et al., 1994; and see later figure).

LY294002, a structurally unrelated and competitive inhibitor of PI 3-kinase (Vlahos et al., 1994), also was tested. LY294002, like wortmannin, inhibited secretion in a concentration-dependent manner, with thapsigargin-induced secretion being the most affected and carbachol and A23187-induced secretion being the least affected (fig. 2B). Also, like wortmannin, LY294002 was an equally potent inhibitor of thapsigargin-induced activation PLD and secretion (table 1). The inhibitory potencies (IC50) of both compounds were in the range of those reported for inhibition of PI 3-kinase in RBL-2H3 cells (table 1) and other types of cells (e.g., Vlahos et al., 1994; Vemuri et al., 1996; Kimura et al., 1994; Okada et al., 1994).

Inhibition of secretion by butanol and propranolol.

To further evaluate the potential role of PLD in mediating secretion, we examined the effects of butanol and propranolol. The first agent is a substrate for transphosphatidylolation by PLD and inhibits formation of phosphatidic acid and secretion (Yang et al., 1967; Stutchfield and Cockcroft, 1993; Perkins et al., 1995), and the second inhibits phosphatidate phosphohydrolase and, thereby, the generation of diglycerides from phosphatidic acid (Lin et al., 1991a). Butanol inhibited the secretory responses to antigen, carbachol, A23187 and thapsigargin (fig. 2C); the values for IC50 for this inhibition varied from 18 to 37 mM for the different stimulants, with 100% inhibition at 100 mM butanol for all stimulants. However, the effects of butanol were complex. Although butanol inhibited antigen-induced hydrolysis of inositol phospholipids (IC50 ~ 50 mM), it did not inhibit carbachol-induced hydrolysis of these phospholipids at concentrations up to 100 mM (data not shown). Secretion was inhibited also by propranolol but to varying extents (fig. 2D) with a rank order of thapsigargin, A23187, antigen and carbachol (91 ± 5%, 81 ± 7%, 72 ± 11% and 36 ± 5% inhibition, respectively, at 250 μM propranolol in three experiments).

The foregoing experiments indicated that although all secretagogues were sensitive to the inhibitory actions of wortmannin, butanol and propranolol, thapsigargin was particularly sensitive. The data were consistent with the notion that PLD mediated secretion, but the known effects of these compounds on PKC and the effects noted here on PLC activity required more rigorous examination of this notion. Thapsigargin was chosen for further study because of its sensitivity to the inhibitors and its minimal stimulation of PLC.

Secretory response to thapsigargin is dependent on rise in [Ca2+]i, and activation of a cholera toxin-sensi-

| TABLE 1 |
| Comparison of inhibitory potencies of the PI 3-kinase inhibitors wortmannin and LY294002 on PI 3-kinase activity and thapsigargin-induced activation of PLD and hexosaminidase secretion in RBL-2H3 cells |
| IC50 |
| PI 3-kinase |
| PLD |
| Secretion |
| Wortmannin (nM) |
| LY294002 (μM) |
| 3 |
| 2 |
| 21 |
| 3.7 |
| 23 |
| 1.6 |

legend: Minutes

Fig. 2. Suppression of secretion by wortmannin, butanol and propranolol. The indicated concentrations of wortmannin (A), LY294002 (B), butanol (C) and propranolol (D) were added to RBL-2H3 cultures before stimulation with 20 ng/ml DNP-BSA (Ag), 1 mM carbachol (CBC), 150 nM thapsigargin (Tg) or 500 nM calcium ionophore (A23187) for 15 min. Release of hexosaminidase from cells was determined as described in Materials and Methods. For A, values represent the mean ± S.E.M. for three separate experiments. For B and C, values (mean ± S.E.M. for three cultures) were from one of two similar experiments. Values were expressed as a percentage of normal response in the absence of inhibitor. Hexosaminidase release in the absence of inhibitors was 37 ± 3%, 24 ± 3%, 16 ± 2% and 25 ± 3% for antigen-, carbachol-, thapsigargin- and A23187-stimulated cells, respectively.
tive PLD. Low concentrations of thapsigargin (1–10 nM) failed to activate PLD or stimulate secretion but, as in previous studies (Ali et al., 1994), caused substantial increases in \([Ca^{2+}]_i\). As little as 5 nM thapsigargin caused increases in \([Ca^{2+}]_i\) > 2 \(\mu M\) in excess of those induced by maximally stimulating cells with antigen or carbachol (data not shown but similar to previous data; see Ali et al., 1994). These concentrations of thapsigargin caused no detectable formation of \(\text{[H]}\)phosphatidylethanol and secretion. Such responses were observed at concentrations of \(>30\ nM\) thapsigargin, and at 300 nM thapsigargin such responses were similar to those evoked by maximally stimulating cells with antigen (10 ng/ml DNP-BSA; fig. 3). Thapsigargin, however, failed to elicit substantial hydrolysis of inositol phospholipids compared with the response evoked by antigen (fig. 3, side). Even though the increase in \([Ca^{2+}]_i\] was apparent with concentrations of thapsigargin that failed to elicit other responses, the activation of PLD and secretion were still dependent on a rise in \([Ca^{2+}]_i\]. Thapsigargin failed to activate PLD or induce secretion (fig. 3, inset) when intact cells were depolarized with high K\(^+\) to block entry of external Ca\(^{2+}\) ions or when cells were permeabilized and \([Ca^{2+}]_i\] was buffered at 1 \(\mu M\) (see Materials and Methods for details). The same permeabilized cells nevertheless exhibited increased PLD activity (0.35 \(\pm\) 0.01% release of \(\text{[H]}\)phosphatidylethanol) and secretion (22 \(\pm\) 1% release of hexosaminidase) when stimulated with antigen (10 ng/ml DNP-BSA; data from three experiments).

We next examined the effects of cholera toxin on thapsigargin-induced responses because of its ability to enhance activation of PLD in stimulated cells. The responses measured included formation of inositol phosphates, \(\text{[H]}\)phosphatidylethanol and release of arachidonic acid to assess activation of PLC, PLD and PL\(\alpha\), respectively, as well as secretion. Treatment with the toxin resulted in markedly enhanced secretory (fig. 4) and PLD (fig. 4, inset) responses to thapsigargin without affecting responses mediated by PLC and PL\(\alpha\) (fig. 4). These results indicated that of the three phospholipases examined, the toxin treatment specifically targeted PLD. Moreover, the extent of activation of PLD and secretion were highly correlated (\(P < .001\)) when data from both untreated and toxin-treated cells were analyzed collectively. This correlation suggested a close association between these two events.

**Dependency of thapsigargin-induced secretion on PKC.** Diglycerides, whether generated through PLD and phosphatidate phosphohydrolase or through PLC, are thought to activate PKC (Asaoka et al., 1992). Ro31-7549, a specific inhibitor of calcium-dependent and independent isoforms of PKC (Wilkinson et al., 1993) in RBL-2H3 cells (Ozawa et al., 1993; Yamada et al., 1992), inhibited thapsigargin-induced secretion, as well as the secretory responses to other stimulants (fig. 5A). It should be noted that the apparent resistance of the secretory responses to antigen and carbachol to low concentrations of this inhibitor (fig. 5A) can be attributed to alleviation of feed-back inhibition of PLC by PKC with these two stimulants. In contrast to Ro31-7549, G6-6976, which selectively inhibits the calcium-dependent isoforms of PKC (Martiny-Baron et al., 1993), had little or no anti-secretagogue activity in thapsigargin-stimulated cells (fig. 5B). The studies in total indicated that secretory responses to thapsigargin were mediated through PKC, most likely through calcium-independent isoforms of PKC.

**Reversal of inhibitory effects ofwortmannin and butanol by PMA.** The possibility that wortmannin and butanol inhibited secretion by suppressing the generation of diglycerides via PLD, and hence the activation of PKC, was examined by activating PKC directly with PMA. The presence of phorbol ester largely reversed the inhibition of secretion by wortmannin (fig. 6) and substantially reversed the inhibition by butanol (fig. 7) in thapsigargin-stimulated cells. The relatively high concentrations of organic vehicle that were required for these experiments may account for the incomplete reversal of the response in the presence of butanol. The reversal was apparent for a wide range of concentrations of thapsigargin when secretion was inhibited by wortmannin (fig. 6) or butanol (fig. 7) even though production of phosphatidic acid remained totally suppressed in the presence of thapsigargin.

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**Fig. 3.** Dependency of secretory response to thapsigargin on PLD and elevated \([Ca^{2+}]_i\]. \(\text{[H]}\)Myristate-labeled RBL-2H3 cells were stimulated with the indicated concentrations of thapsigargin or 10 ng/ml DNP-BSA (Antigen) for 15 min. Release of hexosaminidase from cells (\(\triangle\)) and increase in levels of \(\text{[H]}\)phosphatidylethanol (\(\bigtriangledown\)) were determined as described in Materials and Methods. Values are mean \(\pm\) S.E.M. for three or four separate experiments. Inset, comparison of the release of hexosaminidase (Hexos.) and increase in \(\text{[H]}\)phosphatidylethanol (PETOH) in intact, permeabilized and depolarized (high K\(^{+}\)) cells in response to 300 nM thapsigargin. For these experiments, cells were left intact, permeabilized with streptolysin-O in the presence of buffered 1 \(\mu M\) \([Ca^{2+}]_i\] or exposed to high K\(^{+}\) to block entry of Ca\(^{2+}\) and elevation of \([Ca^{2+}]_i\) as described in text. Values were from one of three similar experiments.
ence of PMA (compare insets in fig. 7, A and B). The reversal by PMA was blocked by the PKC inhibitor Ro31-7549 (10 μM) in the presence of wortmannin (1 ± 0.5% versus 31 ± 2% secretion in the absence of Ro31-7549) or butanol (3 ± 2% versus 27 ± 1% secretion in the absence of Ro31-7549), an indication that secretion was still dependent on PKC (cells were exposed to 150 nM thapsigargin, 50 nM PMA and 100 nM wortmannin or 50 mM butanol). These results suggested that PKC activation was downstream of wortmannin- and butanol-sensitive events. They also suggested that activation of PKC by PMA could substitute for activation of PKC through PI 3-kinase and PLD in providing a synergistic signal for secretion in the presence of a thapsigargin-induced increase in [Ca\(^{2+}\)].

It should be noted that PMA potentiated the secretory response to thapsigargin in the above experiments (compare B with A in figs. 6 and 7). PMA, by itself, activates PLD\(^3\) and both calcium-dependent and -independent isoforms of PKC, but it does not induce secretion in RBL-2H3 cells (Ozawa et al., 1993). If thapsigargin acts largely or exclusively through calcium-independent isoforms of PKC, as suggested by the data in figure 5 (see also Discussion), the potentiation of secretion might reflect recruitment of additional isoforms of PKC by PMA. Nevertheless, the studies with Ro31-7549 indicate that the common denominator of secretion was PKC regardless of the combination of stimulants and inhibitors.

**Discussion**

Secretion in RBL-2H3 cells is dependent on influx of external calcium and rise in [Ca\(^{2+}\)]\(_i\) (Choi et al., 1993; Ali et al., 1994), although activation of PKC is a necessary synergizing signal for secretion in these cells (Sagi-Eisenberg et al., 1985; Ozawa et al., 1993). Here, we demonstrate that activation of PKC is most likely accomplished exclusively through stimulation of PLD in cells stimulated by thapsigargin. Unlike stimulation of RBL-2H3 cells via receptor ligands, thapsigargin elicits minimal activation of PLC (figs. 3 and 4) while markedly activating PLD. This activation of PLD is selectively enhanced in chola toxin-treated cells as has been noted for other stimulants.\(^3\) The close correlation between activation of PLD and secretion and the selective enhancement of these two responses to thapsigargin by cholera toxin (fig. 4) strongly suggests that these two events are closely related. Two additional findings add to this scenario. First, the inactivity of thapsigargin when the increase in [Ca\(^{2+}\)]\(_i\) is suppressed, either by blocking calcium influx in intact cells or by permeabilizing cells (fig. 3, inset), indicates that activation of the toxin-sensitive PI 3-kinase-regulated PLD is secondary to the rise in [Ca\(^{2+}\)]\(_i\). Second, the inhibition of secretion by Ro31-7549 but not by Go-6976 suggest that PLD-generated lipids lead to secretion through calcium-independent isoforms of PKC. The following sequence is indicated. The calcium-mediated activation of PLD provides the primary, and perhaps exclusive, source of diglycerides, via phosphatidate phosphohydrolase, for activation of PKC, which in turn synergises a thapsigargin calcium signal for secretion.

Previous studies have indicated that phosphatidicholine is the major substrate for PLD in stimulated mast cells (Dinh and Kennerly, 1991) and RBL-2H3 cells (Lin et al., 1991a), and consistent with studies in other types of cells (Liscovitch and Cantley, 1994; Billah, 1993), PLD appears to be responsible for the bulk of the diglycerides that are generated during mast cell stimulation. The mechanisms of activation and identities of PLD, however, are not totally defined (Nishizuka, 1995). PLD activities have been described in various types of cells that are regulated by small G proteins such as the ADP-ribosylation factor, ARF and Rho (reviewed in Exton, 1997). These activities are dependent on GTP and PI-4,5-bisphosphate and can be activated by guanosine-5’-O-(3-thio)triphosphate in the presence of these regulators. A mammalian isoform of this type of PLD, PLD1, has been cloned, and it thought to regulate protein trafficking and secretion via Golgi (Hammond et al., 1995, 1997). Another

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**Fig. 4.** Effects of cholera toxin treatment on responses to thapsigargin. RBL-2H3 cells were treated with (closed symbols) or without (open symbols) cholera toxin for 4 hr before stimulation with the indicated concentrations of thapsigargin for 15 min. Release of hexosaminidase (Hex.) and arachidonic acid (AA) from cells and generation of inositol phosphates (IP) were determined as described in Materials and Methods. For clarity, values for release of inositol phosphates were not corrected for spontaneous release. Values (mean ± S.E.M.) were from one of three similar experiments. Inset, data from another experiment that compares the effect of cholera toxin on increases in levels of \([^{3}H]\)phosphatidylethanol (PEtOH) and hexosaminidase release \([^{3}H]\)myristate-labeled RBL-2H3 cells in response to various concentrations of thapsigargin. The effects of cholera toxin were significant (P < .01) for all doses of thapsigargin of >10 nM. Values were from one of three similar experiments.
isoform, PLD2, is not regulated by the small G proteins, and its function remains undetermined (Colley et al., 1997; Kodaki and Yamashita, 1997). As noted earlier, RBL-2H3 cells contain a cholera toxin-sensitive PLD, which, as the current results demonstrate, may generate mediators for secretion in response to cell stimulation. PLD can also be stimulated in permeabilized RBL-2H3 cells, as in other types of cells (see Exton, 1997), by guanosine-5'-O-(3-thio)triphosphate, but this stimulation is dependent on small monomeric G proteins, and it is insensitive to the cholera toxin. We are currently investigating whether the synergistic effect of cholera toxin on PLD activation in RBL-2H3 cells is indicative of a previously undescribed route of activation of PLD or the presence of a PLD isoform other than PLD1.3

Although none of the available inhibitors of PLD-mediated reactions are selective, the patterns of inhibition provide evidence that a toxin-sensitive PLD promotes a robust signal for secretion in thapsigargin-stimulated cells. For example, butanol, a presumed inhibitor of phosphatic acid formation, and propranolol, a presumed inhibitor of diglyceride formation, inhibited secretion. Both these agents, however, inhibit PKC (Sozzani et al., 1992; Slater et al., 1993). Therefore, the effects of these inhibitors alone do not establish that PLD, in conjunction with phosphatidate phosphohydrolase, is essential for secretion. We note, nevertheless, that the anti-secretagogue activities of the inhibitors is most apparent with thapsigargin (fig. 2) and, in the case of wortmannin and butanol, can be substantially reversed by PMA (figs. 6 and 7). The data, at the very least, suggest that these inhibitors do not impair secretion by acting as cell toxicants but rather by preventing activation of PKC. Thapsigargin is known to cause translocation of the ε isozyme of PKC exclusively in RBL-2H3 cells (Wolfe et al., 1996) in contrast to antigen, which induces translocation of most isozymes of PKC (Ozawa et al., 1993). Although thapsigargin-induced secretion is inhibited by Ro31-7549 (current results), which inhibits both calcium-dependent and -independent isozymes of PKC (Wilkinson et al., 1993), this secretion is not inhibited by selective inhibition of the calcium-dependent isozymes of PKC. It is possible, therefore, that PLD-derived lipid metabolites can selectively stimulate PKCe.

Wortmannin has been used to inhibit PLD (Reinhold et al., 1990; Bonser et al., 1991), but whether it does so directly or indirectly has not been determined. Wortmannin is now used...
primarily as an inhibitor of PI 3-kinase. By binding to the p110 catalytic subunit of PI 3-kinase, it irreversibly inactivates the enzyme at nanomolar concentrations (Yano et al., 1993). The suppression of thapsigargin-induced activation of PLD by wortmannin and LY294002 at concentrations known to inhibit PI 3-kinase in RBL-2H3 cells (table 1) and other types of cells (10–50 nM) (Vemuri et al., 1996; Kimura et al., 1994; Okada et al., 1994), raises the question of whether PI 3-kinase is required for activation of the toxin-sensitive PLD. The inhibitory potencies (IC50) of wortmannin and LY294002 at concentrations known whether PI 3-kinase is required for activation of the toxin-sensitive PLD is not only essential for mediating PKC-dependent secretion in response to thapsigargin but also may participate in the secretory process in response to other secretagogues.

In conclusion, the present results define a PLD-dependent pathway for stimulation of secretion in response to thapsigargin. It is clear that secretion is dependent not simply on the elevation of \([Ca^{2+}]_i\) but also on the secondary activation of phospholipases by elevated calcium. Of these phospholipases, PLD appears to be the primary enzyme for the generation of diglycerides for activation of PKC and secretion. On the basis of the marked enhancement of PLD activity by cholela toxin, we conclude that the PLD activated by thapsigargin is a cholela toxin-sensitive PLD. The present and ongoing studies suggest that activation of a cholera toxin-sensitive and PI 3-kinase-regulated PLD is not only essential for mediating PKC-dependent secretion in response to thapsigargin but also may participate in the secretory process in response to other secretagogues.

References


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