Activation of phospholipase D-2 by $P2X_7$ agonists in rat submandibular gland acini

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Abstract **Exogenous ATP stimulated phospholipase D (PLD), but not sphingomyelinase in rat submandibular gland (SMG) acini. PLD activation was dependent upon extracellular Ca^{2+} and did not involve intracellular Ca^{2+} mobilization or phosphoinositide-specific phospholipase C activation. ATP-stimulated PLD was attenuated by inhibition or downregulation of protein kinase C (PKC). PLD activation was fully blocked by the cytosolic phospholipase A_2 (PLA_2) inhibitor ONO-RS-082 and partially attenuated by the selective Ca^{2+}-dependent cytosolic PLA_2 inhibitor, arachidonyl trifluoromethylketone ($AACOCF_3$), or by bromoenol lactone, an inhibitor of Ca^{2+}-independent cytosolic PLA_2. Magnesium, which decreases the concentration of ATP^{4-}, and nickel, which blocks nonspecific cation channels coupled to purinergic receptors, inhibited PLD activation by ATP. Using reverse transcription-polymerase chain reaction and Northern blotting techniques, we demonstrated that the PLD isoform stimulated by ATP was PLD-2. Among various ATP analogs, only the P2Z/$P2X_7$ purinergic receptor agonist benzoyl-benzoyl ATP stimulated PLD-2. The response to ATP was inhibited by the nonselective P2X purinergic antagonist suramin and by oxidized ATP, a potent P2Z/$P2X_7$ receptor antagonist. It is concluded that in rat SMG acinar cells, PLD-2 is upregulated by exogenous ATP through a mechanism involving Ca^{2+} influx, cytosolic PLA_2, and PKC. Also, the data suggest an involvement of $P2X_7$ receptors in PLD-2 stimulation by ATP.**—Pérez-Andrés, E., M. Fernández-Rodriguez, M. González, A. Zubiaga, A. Vallejo, I. García, C. Matute, S. Pochet, J. P. Dehaye, M. Trueba, A. Marino, and A. Gómez-Muñoz. **Activation of phospholipase D-2 by $P2X_7$ agonists in rat submandibular gland acini. *J. Lipid Res.* 2002.** 43: **1244–1255.**

Supplementary key words ATP • calcium • ceramides • phospholipase A_2 • phospholipase C • phospholipase D • protein kinase C • purinergic receptors • sphingosine 1-phosphate

Extracellular ATP can regulate a wide range of physiological functions. In most cells, the cytoplasmic concentration of ATP is in the millimolar range, but in storage granules of platelets, the concentrations of ATP and ADP can be as high as 1 M (1). Under conditions of platelet activation or cell breakage, significant portions of ATP can be released into the extracellular environment so that the concentrations of pericellular ATP can easily reach the high micromolar range. The effects elicited by ATP appear to be mediated through specific P2-purinergic receptors (2). These receptors are currently classified into two major groups. The first group includes the metabotropic P2Y receptors, which are proteins with seven transmembrane domains that are coupled to guanine 5′-triphosphate (GTP) binding proteins (G-proteins). Agonist stimulation of P2Y receptors causes intracellular Ca^{2+} mobilization through the hydrolysis of phosphoinositides (PI) by phospholipase C (PLC) (3, 4). The second group includes the ionotropic P2X receptors, which have two transmembrane domains. Agonist stimulation of these receptors induces the formation of nonspecific cation channels (5–8). Up to now, seven P2Y receptors and eight P2X receptors have been cloned (9–11). Among the P2X receptors, the $P2X_7$ receptor is unique in that its activation leads not only to the formation of a nonspecific cation channel but also to large pores. It was previously termed P2Z and therefore it is known as the P2Z/$P2X_7$ receptor

Abbreviations: $AACOCF_3$, arachidonyl trifluoromethylketone; BEL, (E)-6-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (bromoenol lactone); Bz-ATP, 2′,3′-*O*-(benzoyl-benzoyl)adenosine 5′-triphosphate; $cPLA_2$, calcium-dependent cytosolic phospholipase A_2; DAG, diacylglycerol; HBS, HEPES-buffered saline; $iPLA_2$, calcium-independent cytosolic phospholipase A_2; PA, phosphatidic acid; PC, phosphatidylcholine; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13 acetate; RT-PCR, reverse transcription-polymerase chain reaction; SMG, submandibular gland.

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(7, 11). Although ATP can increase the permeability of cells to a variety of cations, including Na^+, K^+, or Ba^{2+}, the channel shows considerable selectivity for Ca^{2+} (3). The stimulation of P2Z/$P2X_7$ receptors leads to phospholipase D (PLD) activation in human lymphocytes (3) and in osteoblast-like cells (12). Activation of PLD is of particular interest because it increases the intracellular concentration of phosphatidic acid (PA), which is bioactive (13–15). In addition, PA is the precursor of important second messengers, as it can give rise to lysoPA by the action of PLA_2, or to diacylglycerol (DAG) when it is acted upon by phosphatidate phosphohydrolases. Two isoforms of mammalian PLD have been identified in mammalian cells and then cloned. PLD-1 can be activated by monomeric G-proteins of the ADP-ribosylation factor (ARF) and Rho families, and protein kinase C (PKC), whereas PLD-2 shows little or no response to the known activators of PLD-1 (16). There is also evidence for the existence of a distinct PLD that is activated by oleate (16, 17). However, cloning of the oleate-dependent PLD isoform has not yet been reported.

The present study was undertaken to investigate which of the known PLDs was sensitive to activation by ATP, and to establish the underlying mechanism(s) for this activation in submandibular gland (SMG) acini. The possible involvement of P2Z/$P2X_7$ receptors in this process was also examined.

MATERIALS AND METHODS

Materials

ATP (sodium salt), adenosine-5′-*O*-(3-thiotriphosphate (ATPγS), BSA (BSA, fraction V), and collagenase P were from Boehringer Mannheim (Mannheim, Germany). 2,3-Dialdehyde ATP (periodate-oxidized ATP). Adenosine 5′-diphosphate (ADP), benzoyl-benzoyl ATP (Bz-ATP), cytidine 5′-triphosphate (CTP), digitonin, DNase I (from bovine pancreas), EGTA, GTP, HEPES, PA, phosphatidylcholine (PC), 4β-phorbol 12-myristate 13 acetate (PMA), sphingomyelinase (from *Staphylococcus aureus*), uridine 5′ triphosphate (UTP), αβ-methylene ATP (αβ-mATP), mega-10 detergent, and fatty acid standards were from Sigma (St. Louis, MO). [^{3}H]myristic acid (60 Ci/mmol) was supplied by American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]oleic acid (5 Ci/mmol) was from NEN (Boston, MA). Percoll was from Pharmacia (Uppsala, Sweden). The Dowex AG1-X8 columns were from Bio-Rad (Hercules, CA). Phosphatidylethanol standard was supplied by Avanti Polar-lipids (Alabaster, AL). Fura-2-acetoxymethylester (AM) was from Molecular Probes (Eugene, OR). Suramin was supplied by Alexis Biochemicals (Woburn, MA). Chelerythrine was from Calbiochem (La Jolla, CA). C_2-ceramide was from Matreya, Inc. (Pleasant Gap, PA). The thin layer chromatography (TLC) plates of silica gel 60 were from Macherey-Nagel (Düren, Germany). Other chemicals were the highest grade available and their sources have been described (8).

Preparation of SMG acini

Acinar cells were isolated from male Sprague-Dawley rats (200–250 g) that were given free access to water and food. The rats were killed by exposure to diethylether atmosphere. SMGs were then immediately dissected and minced in about 0.2 ml of HEPES-buffered saline (HBS) containing 96 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 11 mM glucose, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 1% (v/v) glutamine-free amino acid mixture (Life Technologies, Inc.), and 24.5 mM HEPES. The pH was adjusted to 7.4 with NaOH. The medium was supplemented with 0.125% (w/v) BSA. A homogeneous suspension of acinar cells was obtained essentially as described (8) with minor modifications. The minced tissue was then incubated in 10 ml HBS medium containing 1.5 mg of collagenase P (1.52 U/mg) at 37°C for 20 min under constant shaking (about 90 cycles/min). After the first 10 min of incubation, the tissue was aspirated 10 times with a 10 ml glass pipette. This process was repeated at the end of the incubation time and was followed by aspiration of the cell suspension with 5 ml and 2 ml glass pipettes. The cell suspension was then washed three times with an isotonic saline solution, resuspended in 10 ml of collagenase-free HBS and further incubated for 10 min in the presence of 0.06 mg/ml of DNAase I (420 U/mg). The crude suspension was aspirated again several times with a 2 ml glass pipette, filtered through a nylon mesh, and washed three times with saline solution. To separate the acini from the ducts, the cell suspension was resuspended in 4 ml of HBS and distributed in two tubes containing 6 ml of an isotonic Percoll solution (40%). The cell suspension was carefully layered on top of the Percoll solution. The tubes were then centrifuged at 4,000 *g* at 4°C for 10 min. The acini sedimented through the Percoll solution, whereas ductal cells remained on the top. The acinar cells were then recovered and washed three times with the isotonic saline solution. The final pellet was resuspended in HBS medium and kept at 4°C for 10 min before being used in the experiments.

Cell culture

Raw 264.7 macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) containing penicillin and streptomycin supplemented with 10% fetal bovine serum (FBS). Then, they were washed three times in DMEM, preincubated for 1 h in DMEM containing 1% FBS with or without inhibitors prior to addition of agonists, and then incubated for the appropriate times before being harvested.

Assay of PLD activity

PLD was determined in vivo by measuring the production of [^{3}H]phosphatidylethanol, which is the product of its transphosphatidylation activity when cells containing [^{3}H]PC are incubated in the presence of ethanol (18). Rat SMG acini were incubated for 2 h with HBS medium containing 0.125% BSA, 0.5 mM Ca^{2+}, and 10 μCi of [^{3}H]myristate/ml to label cell PC. The radioactive medium was then removed by centrifugation and the cells washed three times with isotonic saline solution and resuspended in HBS medium with 1.5 mM Ca^{2+}. Ethanol, at a final concentration of 1%, was added 5 min prior to the addition of agonists and the cells incubated at the times indicated. This concentration of ethanol gave maximal formation of phosphatidylethanol, as shown in the results section, with no toxicity for the cells. The acini were incubated with the agonists for varying times at 37°C with constant shaking (about 90 cycles/min) in plastic vials in a final volume of 400 μl. Aliquots (100 μl) were then withdrawn from each vial in triplicate and lipids were extracted as described by Bligh and Dyer (19), except that 2 M KCl in 0.2 M HCl was added to the extraction mixture instead of water for separation of phases. Chloroform phases were then vacuum-dried in an automatic Speed Vac concentrator (Savant AS290) and resuspended in 50 μl chloroform. Lipids were separated by TLC using 20 × 20 cm silica gel 60-coated aluminum plates. TLC plates were developed for 50% of their lengths with chloroform-methanol-acetic acid (9:1:1, v/v/v) and then dried.

The plates were then redeveloped for their full lengths with petroleum ether-diethylether-acetic acid (60:40:1, v/v/v). The position of the lipids was identified after staining with I_2 vapor by comparison with authentic standards. The silica gel containing radioactive lipids was quantitated by liquid scintillation counting after scraping from the plates.

The effect of GTPγS and recombinant ARF1 on PLD was examined in cells permeabilized by treatment with digitonin (50 μg/ml) in a final concentration of 1% ethanol according to Li et al. (20).

To assay the oleate-dependent PLD activity in vitro, we first obtained purified PC from SMG acini that were labeled with [^{3}H]myristate (20 μCi/ml) for 4 h. The lipids were extracted as indicated above. [^{3}H]PC was purified by TLC on silica gel 60 using chloroform-methanol-water (65:35:7, v/v/v) and visualized by iodine vapor. [^{3}H]PC was eluted from the TLC plates by washing the silica gel three times with chloroform-methanol-acetic acid-water (50:39:1:10, v/v/v/v). Eluates were combined and phases separated by adding 1 ml of water. The organic phase containing the [^{3}H]PC was washed twice with 4 ml of methanol-water (1:1, v/v) and then dried. Assays were performed essentially according to Lee et al. (17). Briefly, the assay mixture consisted of 60 μM [^{3}H]PC (80,000 dpm), 1 mM PA, 2.5 mM oleate, 10 mM $CaCl_2$, 25 mM potassium fluoride, 2.5 mM $MgCl_2$, 0.05% (w/v) Mega-10, 1.5% ethanol, 50 mM HEPES buffer (pH 7.0) and 100–200 μg of homogenate or microsomal or cytosolic fractions in a total volume of 100 μl. The reaction mixture was incubated for 90 min at 30°C. The lipid extraction and measurement of [^{3}H]phosphatidylethanol radioactivity was performed as described above.

Assay of phosphatidylinositol-specific phospholipase C activity

Phosphatidylinositol-specific phospholipase C (PI-PLC) was determined essentially as described (21). Briefly, SMG acinar cells obtained from one rat were washed with 10 ml of an isotonic saline solution and resuspended in 3 ml of HBS containing 1% BSA and 1 mM Ca^{2+}. Cells were then incubated for 90 min at 25°C in the presence of 25 μCi/ml of *myo*-[2-^{3}H]inositol to label cell phosphoinositides. The acini were then washed twice with 10 ml of HBS in the absence of radioactive inositol and pre-incubated in 4.5 ml of HBS containing 0.125% BSA and 1 mM Ca^{2+} for 10 min at 37°C in the presence of 10 mM LiCl. Aliquots (100 μl) were incubated in a final 500 μl of HBS medium in the presence or in the absence of ATP for 10 min. Samples were centrifuged at 10,000 *g* for 30 s. Supernatants were discarded, and 500 μl of 10% (w/v) ice-cold trichloroacetic acid were added to the pellet. Samples were spun down for 2 min at 10,000 *g*, and the supernatants were transferred to glass tubes. The pellets were washed again with 500 μl of 10% trichloroacetic acid, centrifuged, and then pooled. Trichloroacetic acid was then extracted with water-saturated diethylether. The extracts were neutralized with 1 M KOH and diluted with 8 ml of 10 mM HEPES and 2 mM EDTA (pH 7.4). Inositol phosphates were isolated by using Dowex AG1-X8 columns as described previously (21). The radioactive samples eluted off the column were quantified by liquid scintillation counting.

Measurement of Ca^{2+} concentrations

Intracellular Ca^{2+} concentration was determined as described previously (22). Briefly, 1 ml aliquots of acini suspensions were incubated at 25°C with 1 ml HBS buffer containing 0.125% (w/v) BSA, 0.5 mM $CaCl_2$, and 2 μM Fura-2/AM. After 45 min, 1 ml of this suspension was withdrawn, washed with isotonic saline, and resuspended in 2 ml HBS without amino acids or BSA, and 1 mM Ca^{2+}. Cells were constantly stirred in the cuvette of a fluorimeter (SLM Aminco Bowman Series 2). The excitation wavelength was switched every second from 340 nm to 380 nm (slit width 4 nm), and the light emitted at 510 nm (slit width 4 nm) was recorded. The voltage of the photomultiplier was 700–750 V. At the beginning of each assay, the signal observed after excitation at 340 nm was arbitrarily set at 50% maximal scale by adjusting the voltage. At the end of each assay, the traces were calibrated with the successive addition of 0.5 mM digitonin and 40 mM EGTA in Tris buffer (pH 8.5). Autofluorescence was measured in the presence of $NiCl_2$ (250 mM). The values obtained were subtracted from all the data before calculation of ratios. Ca^{2+} concentrations were estimated by the ratio method (21), using a K_d value of 263 nM.

RNA extraction and reverse transcription-polymerase chain reaction

Acinar and ductal cells were purified through a Percoll gradient three times in order to minimize cellular contamination. Total RNA was extracted from cellular fractions with TRIzol Reagent (Invitrogen), based on the Chomczynski and Sacchi method (23), according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of RNA using SuperScript First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR), and amplification reactions were performed with Platinum PCR Supermix (Invitrogen) in accordance with the supplier's instructions. Specific primers for detection of P2Z/$P2X_7$ transcripts were designed exactly as described previously (8). Sequences of primers used for the RT-PCR detection of PLD isoforms (Genset, FR) and amplification conditions for PLD-1b and PLD-2 were performed according to Gibbs and Meier (24). PLD-1a primers were designed using the Genetics Computer Group program GCG Primer taking into account the difference of 38 amino acids with its splice variant (see sequences below). Conditions for PCR were: 94°C for 1 min, 56°C for 1.5 min, and 72°C for 2 min for 30 cycles (PLD-1a and PLD-1b); 94°C for 1 min, 56°C for 1 min, and 60°C for 1.5 min for 40 cycles (PLD-2). Amplified products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide. In order to test for the presence of contaminating genomic DNA (gDNA), the same cDNA samples were subjected to PCR with β-actin primers (Clontech, CA). Since these primers span three introns, contaminating gDNA can be detected as a 1,440 bp PCR product in addition to a 764 bp product, the latter corresponding to expressed RNA.

Gene and Accession Number (GenBank)	Primers Coordinates	Primer Sequence (5′→3′)	PCR Product (bp)
PLD1a - AB000778	886-905	GGGGGACACAGGATACCAGG	1,159
	2,025-2,044	GGCCCTGCTCAGACTCACTG	
PLD1b - AB000779	871-890	GGGGGACACAGGATACCAGG	1,078
	1,929-1,948	GGATGGAGCCGGTGTTGGAG	
PLD2 - D88672	2,042-2,063	TCAAGGCCAGATACAAGATACC	332
	2,352-2,373	CACGTAGACTCGGAAACACTGC	

Preparation of cDNA probes

RT-PCR products were purified using the Gel Extraction System kit (Invitrogen) after checking their identity with restriction enzymes. Twenty-five nanograms of PLD1a or PLD1b cDNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) using a Random Primed DNA Labeling Kit from Boehringer Mannheim. The radiolabeled probes were purified by chromatography to remove the unincorporated radionucleotides.

Northern blot analysis

RNA samples (40 μg/lane) were denatured with formamide and formaldehyde and size-separated by 1.2% agarose gel electrophoresis in MOPS buffer. The RNAs were blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech), UV-crosslinked, and hybridized by standard methods (25). The expression of each gene was visualized and quantitated by a Molecular Imager FX System and QuantityOne software (Bio-Rad laboratories). Blots were stripped by washing at 65°C for 15 min in TE buffer with 0.1% SDS prior to reprobing.

Measurement of ceramide production

[^{3}H]ceramides were determined by scraping the ceramides from the same thin-layer plate as that used for isolating [^{3}H]phosphatidylethanol and quantifying the radioactive ceramides by liquid scintillation counting, as indicated previously (26). The identity of the ceramide was confirmed by cochromatography with authentic ceramides.

Statistical analysis

Unless stated otherwise, results are expressed as means ± SEM of the number of experiments that are indicated performed in triplicate. Statistical significance of the difference between means of control and experimental conditions was assessed with Student's paired *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

ATP stimulates PLD activity in rat SMG acini

PLD was determined by measuring the accumulation of [^{3}H]phosphatidylethanol as indicated in the Materials and Materials and Methods section. In a first set of experiments, we confirmed that [^{3}H]myristic acid was selectively incorporated into PC, which is the main substrate for PLD in mammalian tissues (16, 27). Maximal incorporation of [^{3}H]myristic acid into PC occurred after about 2 h of incubation with the acini. By contrast, [^{3}H]oleic acid was equally distributed in PC and phosphatidylethanolamine (data not shown). Exposure of [^{3}H]myristic acid-labeled rat SMG acini to exogenous ATP in the presence of 1% ethanol caused a substantial increase in the accumulation of [^{3}H]phosphatidylethanol, which was time and concentration dependent (**Fig. 1**). Maximal PLD activation by ATP was attained after about 1.5 mM. Concentrations of ATP above 10 mM, although not toxic for the acini, were less effective in activating PLD (data not shown). The final concentration of ethanol was kept at 1% in all of the experiments, as this concentration provided maximal fold increase in the accumulation of phosphatidylethanol relative to control values (**Fig. 2**). The extent of PLD activation varied among different experiments, but there was consistency in cell responsiveness for the SMG acini of individual animals. PLD stimulation by ATP was 4.6 ± 0.3 (mean ± SEM of 50 independent experiments performed in triplicate).

An important signaling pathway that could be implicated in the stimulation of PLD is the sphingomyelinase (SMase) pathway. Stimulation of SMase activity generates ceramide and phosphorylcholine from sphingomyelin (SM). Although ceramides are potent inhibitors of PLD

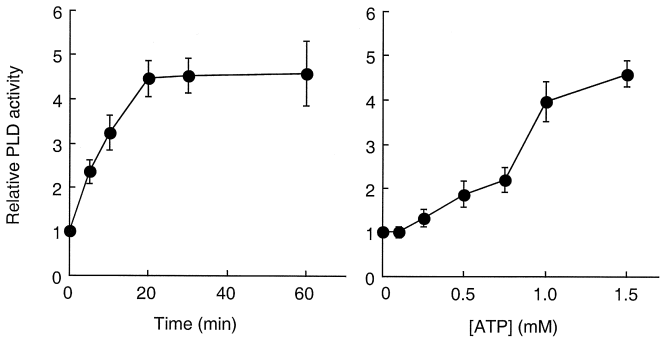

Fig. 1. Stimulation of phospholipase D (PLD) activity by ATP in submandibular gland (SMG) acini. Cells were labeled with [^{3}H]myristate (10 μCi/ml) for 2 h in HEPES-buffered saline (HBS) medium containing 0.125% BSA. Acini were then treated with 1.5 mM ATP for various times, as indicated (left panel) or with increasing concentrations of ATP for 30 min (right panel) in the presence of 1% ethanol. [^{3}H]phosphatidylethanol formation was determined by separating the lipids by thin-layer chromatography (TLC) and processed as described in the Materials and Methods section. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of ATP. For control incubations, typical radioactivity measurements were 495 ± 61 dpm (mean ± SEM, n = 44) per 100 μl cell aliquot in phosphatidylethanol, and 647,595 ± 37,310 dpm (mean ± SEM, n = 44) in total lipids. Results are expressed as the means ± SEM of four independent experiments.

(14, 18, 26, 28–33), they have been shown to stimulate this enzyme activity in human fibroblasts (34). Ceramides can also be generated by de novo synthesis, and could lead to stimulation of PLD via conversion to sphingosine by the action of ceramidases. Sphingosine, in turn, can be phosphorylated to sphingosine 1-phosphate, and both sphingosine and sphingosine 1-phosphate are potent stimulators of PLD in different cell types (13, 26, 34–38). However, ATP did not induce ceramide accumulation when the acini were incubated for up to 3 h in the presence of increasing concentrations of this nucleotide (1–10 mM). In addition, sphingosine failed to stimulate PLD when it was added to the cells at 5 or 10 μM for up to 3 h, and sphingosine 1-phosphate was a weak activator of PLD in the acini. Maximal activation by sphingosine 1-phosphate was attained at 5 μM, and this stimulated PLD by about 1.71 ± 0.15-fold (mean ± SEM of three independent experiments, $P < 0.05$) over the control value. Also, incubation of the acini for up to 3 h with 5–100 μM of the short-chain ceramide *N*-acetylsphingosine ([C_2]ceramide), or with exogenous bacterial SMase (up to 1 U/ml), which generates long-chain ceramides at the plasma membrane of cells, did not induce PLD activation (data not shown). Furthermore, and in agreement with previous work (18, 26), [C_2]ceramide (100 μM, in the presence of 0.125% BSA) inhibited ATP-stimulated PLD by about 77 ± 8% (mean ± SEM of three independent experiments, $P < 0.01$), and pretreatment of the acini for 2 h with exogenous SMase (1 U/ml) resulted in 56 ± 16% (mean ± SEM of four independent experiments, $P < 0.05$) inhibi-

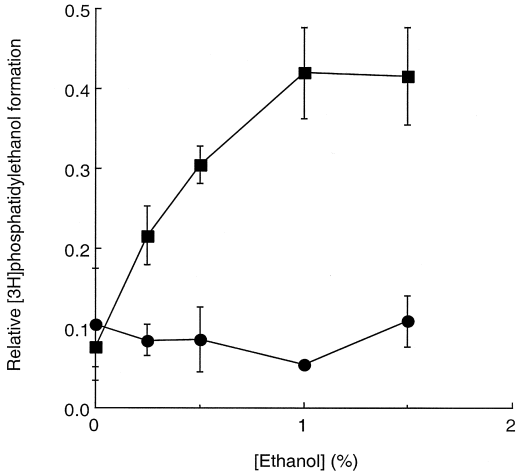

Fig. 2. Effect of increasing concentrations of ethanol on phosphatidylethanol formation by ATP in SMG acini. Cells were labeled and treated as indicated in Fig. 1, except that they were incubated for 30 min in the absence (circles) or presence (squares) of 1.5 mM ATP and increasing concentrations of ethanol, as indicated. Values were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids. Results are the mean ± SEM of three independent experiments.

tion of this enzyme activity. Therefore, it can be concluded that PLD activation by ATP is not mediated through the generation of ceramide or its further metabolism to sphingosine or sphingosine 1-phosphate.

Role of Ca^{2+} in the stimulation of PLD by ATP in SMG acini

ATP can stimulate both intracellular Ca^{2+} mobilization and Ca^{2+} influx. The former action can be induced by relatively low concentrations of ATP (10–100 μM), whereas the latter requires higher ATP concentrations (0.5–1.5 mM) (21, 22). Low concentrations of ATP (10–100 μM) can also increase the levels of inositol trisphosphate (IP_3), suggesting that a phosphatidylinositol-specific phospholipase C (PI-PLC) is involved in this process (21, 22). However, these relatively low concentrations of ATP failed to stimulate PLD in the acini (Fig. 1, right panel). By contrast, 1 mM ATP allowed extracellular Ca^{2+} (kept at 1 mM) to enter the cells, causing a rise in the intracellular concentration of this cation from 85.3 ± 5.6 nM to 175 ± 6.3 nM (mean ± SEM of 10 independent determinations) after 10 s. Interestingly, this higher concentration of ATP failed to stimulate PI-PLC [(21) and unpublished observations]. PLD stimulation by ATP increased with increasing concentrations of extracellular Ca^{2+} (**Fig. 3**), and addition of EGTA (5 mM) to the incubation medium completely blocked this effect. Taken together, these results indicate that the activation of PLD by ATP requires Ca^{2+} influx from the external environment, but is independent of intracellular Ca^{2+} mobilization.

Role of PKC in the stimulation of PLD by ATP in SMG acini

Several lines of evidence indicate that PKC plays a major role in agonist stimulation of PLD activity (16). PMA (100 nM), a well established PKC activator, doubled the activity of PLD in SMG acini, and this stimulation was blocked by the selective PKC inhibitor chelerythrine (**Fig. 4**, right panel). The signaling cascade leading to activation of PLD via PKC usually involves the initial stimulation of PI-PLC isozymes, which hydrolyze phosphatidylinositol bisphosphate to generate DAG and inositol 1,4,5-trisphosphate (IP_3)-mediated release of intracellular Ca^{2+}. However, although we found that concentrations of ATP that are able to activate PLD failed to stimulate the generation of IP_3 or the mobilization of intracellular Ca^{2+} (not shown), PKC may still be involved in PLD activation through mechanisms that are independent of PI-PLC. This possibility was evaluated by downregulating PKC with prolonged incubations of the acini with PMA. Although PKC downregulation is a long-term process, it has been demonstrated that in rat SMG slices, it occurs after 2 h treatment with phorbol esters (39). We observed that after 4 h preincubation with PMA (500 nM), the acini lost their sensitivity to rapid activation of PLD by PMA, and the stimulation of PLD by ATP was markedly reduced (Fig. 4, left panel). Although this inhibitory effect on PLD was incomplete, no further inhibition of this enzyme activity was attained by increasing the incubation time of the acini with PMA for up to 5 h. Incubation times longer than 5 h were not considered for experiments because the viability of the cells started to decrease after this time, as assessed by lactate dehydrogenase release into the medium. Therefore, the role of PKC on ATP-stimulated PLD was further evaluated with chelerythrine. This PKC inhibitor potently blocked the activation of PLD by ATP (Fig. 4, right panel). Similar results were obtained with the selective PKC inhibitor Ro-32-0432 (1 μM) (data not shown). Taken together, these results suggest an involvement of PKC in the stimulation of PLD by ATP in SMG acini.

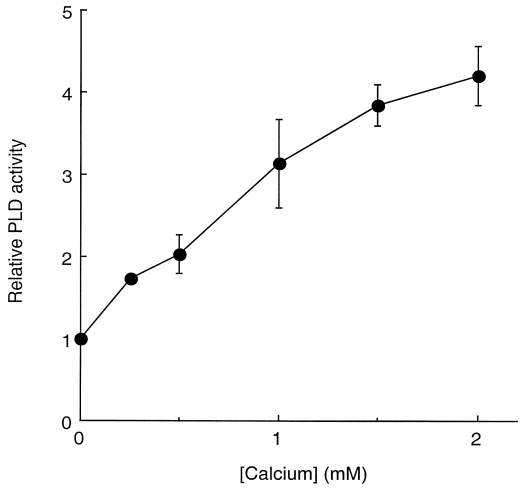

Fig. 3. Effect of extracellular Ca^{2+} on the activation of PLD by ATP in SMG acini. Cells were labeled and treated as indicated in Fig. 1, except that they were stimulated for 30 min with 1.5 mM ATP and 1% ethanol in the presence of increasing concentrations of Ca^{2+}, as indicated. Results are the mean ± SEM of three independent experiments.

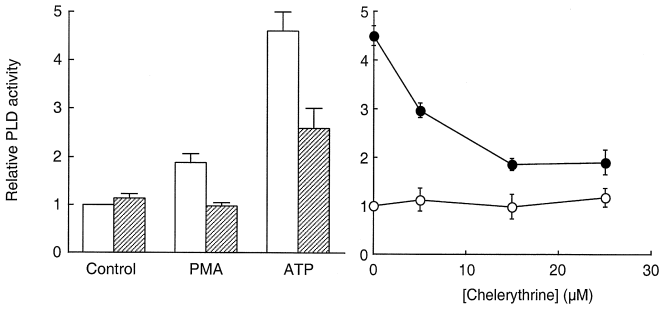

Fig. 4. Involvement of protein kinase C (PKC) in the stimulation of PLD by ATP. The left panel shows the effect of PKC downregulation on ATP-stimulated PLD activation. SMG acinar cells were labeled and treated as indicated in Fig. 1, except that they were preincubated for 4 h in the absence (open bars) or in the presence (hatched bars) of 500 nM 4β-phorbol 12-myristate 13 acetate (PMA) to downregulate PKC activity. Cells were then stimulated with 1.5 mM ATP or 100 nM PMA for 30 min, as indicated. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of ATP or PMA. Results are the mean ± SEM of four independent experiments. The right panel shows the effect of the selective PKC inhibitor chelerythrine on the activation of PLD by ATP. Cells were preincubated for 30 min with the concentrations of chelerythrine that are indicated. The acini were then treated without (open circles) or with 1.5 mM ATP to stimulate PLD and the incubations continued further for 30 min. As expected, 25 μM chelerythrine also inhibited the stimulation of PLD by 100 nM PMA from 2.3 ± 0.1 to 1.4 ± 0.1-fold. The results are the means ± SEM of three independent experiments performed in triplicate.

There is also evidence for an involvement of tyrosine phosphorylation in the regulation of PLD activity [Reviewed by Exton (16)]. In particular, vanadate, which inhibits tyrosine phosphatases, has been shown to stimulate basal PLD potently and to enhance agonist-stimulated PLD activation in different cell types (16, 30, 31, 40–42). However, in SMG acini, tyrosine phosphorylation may not be a major mechanism for PLD activation, as vanadate (200 μM) only activated PLD by about 30 ± 12% (means ± SEM of five independent experiments), and did not enhance the stimulation of PLD by ATP. In addition, the potent tyrosine kinase inhibitor genistein (100 μM) failed to attenuate ATP-stimulated PLD activity (data not shown).

Identification of PLD-2 in rat SMG acini

To identify the PLD isoforms that were susceptible to activation by ATP, purified RNA from SMG acini or ducts was used to specifically amplify PLD-1 and PLD-2 sequences by RT-PCR. Since PLD-1 can be expressed as two splice variants, PLD-1a and PLD-1b, the latter lacking 38 amino acids (43), specific primers were used to amplify each of these transcripts independently. **Figure 5** (upper panel) shows that ductal cells express both PLD-1a and PLD-1b mRNAs, whereas none of these transcripts could be detected in the acini. However, it is noteworthy that RT-PCR reactions carried out with specific primers for PLD-2 revealed that this PLD isoform is expressed in both acinar and ductal cells (Fig. 5, lower panel). To verify the absence of PLD-1 mRNA in SMG acini, we performed Northern blot analysis with cDNA probes specific for PLD-1a and PLD-1b. **Figure 6** demonstrates the absence of PLD-1a and PLD-1b mRNAs in the acini, thereby confirming the results obtained with RT-PCR. By contrast, ductal cells show intense bands for the mRNAs of both PLD isozymes. These observations were further confirmed by using the small GTP binding protein ARF and GTPγS, a nonhydrolyzable analog of GTP, on cytosolic and membrane fractions from cells that were permeabilized with 50 μg/ml of digitonin. Although soluble PLD activities have been reported, in general, mammalian PLDs appear to be membrane-bound enzymes (44). Furthermore, it is well established that ARF is an essential cofactor for activation of PLD-1, but it is not required for activation of PLD-2 (16, 45, 46). In this regard, we show here that ARF stimulated PLD activity in the presence of GTPγS in membrane fractions of ductal cells, which express both PLD-1 and PLD-2, but not in membrane fractions of acini, which only express the PLD-2 isoform (**Fig. 7**). Also, and in agreement with other work (20), no PLD activity was detected in cytosolic fractions of either ductal or acinar cells in the presence of ARF and GTPγS (data not shown). It can then be concluded that ductal cells contain both PLD-1 and PLD-2 isoforms, whereas only PLD-2 is present in the acini.

There is evidence for the existence of another mammalian PLD that is distinct from PLD-1 and PLD-2. This PLD isoform is potently stimulated by oleate, or by other unsaturated fatty acids, although to a lesser extent than by oleate (17, 47). The so-called oleate-dependent PLD has

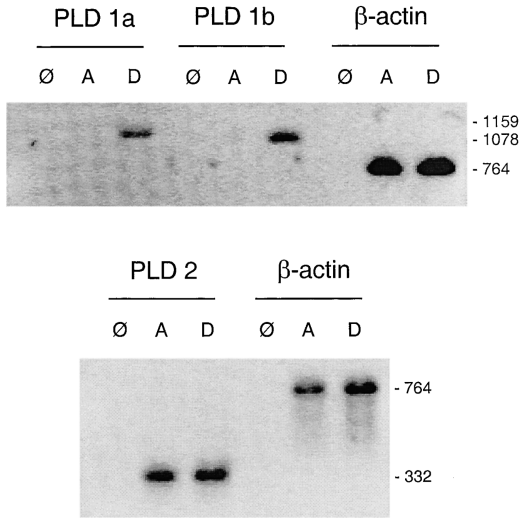

Fig. 5. Identification of PLD isoforms by reverse transcription-polymerase chain reaction (RT-PCR) in rat SMG cells. Total RNA (2 μg) of SMG acini (A), ducts (D), or vehicle (ø) was subjected to RT-PCR, as described in the Materials and Methods section. RT-PCR amplifications of β-actin mRNA were performed in parallel in the same experiments as control to verify the integrity of the cDNA and the absence of contaminating genomic DNA (gDNA). The numbering on the right corresponds to the size in base pairs of the amplicons. Data are representative of three independent experiments.

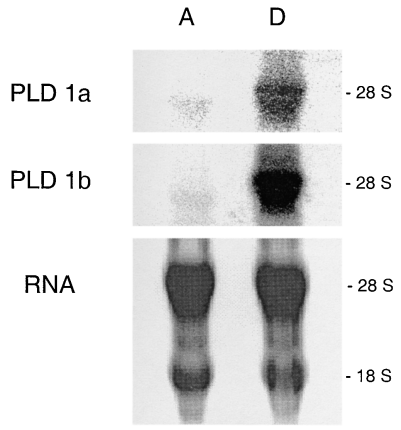

Fig. 6. Northern blots for PLD-1 isoform expression in rat SMG cells. Total RNA (40 μg) of SMG acini (A) and ducts (D) was blotted and sequentially hybridized with radiolabeled probes specific for PLD-1a and PLD-1b mRNAs, as described in Materials and Methods. Ethidium bromide staining of agarose gels (lower panel) demonstrates equal RNA loading in each lane.

been purified from pig lung membranes (48), but to our knowledge it has not been cloned yet. To determine if oleate-dependent PLD activity was present in SMG acini, cells were incubated with increasing concentrations of oleate (0.25–3 mM) for various times up to 2 h. Under these conditions, oleate did not cause any significant accumulation of [^{3}H]phosphatidylethanol, suggesting that SMG acini lack oleate-dependent PLD. Measurements of PLD activity were also conducted in vitro to determine if oleate could stimulate PLD in a cell-free system. Incubation of cell homogenates with exogenous [^{3}H]PC under optimal conditions for measuring oleate-dependent PLD, in the presence of ethanol (1.5%) (17), showed no formation of [^{3}H]phosphatidylethanol, further confirming the lack of this enzyme activity in SMG acini (data not shown). More recently, it has been demonstrated that oleate can either enhance or inhibit PLD-2 activity in different cell types (41, 49). Therefore, we tested to see whether acinar PLD-2 activity could be affected by oleate. **Figure 8** shows that oleate (1 mM) completely blocked the stimulation of PLD by 1 mM ATP or 100 nM PMA in SMG acini. In addition, it was reported (50) that, unlike PLD-1, the activity of PLD-2 is specifically regulated by cytosolic phospholipase A_2 (PLA_2), and we recently demonstrated that ATP stimulates both calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent cytosolic phospholipase A_2 ($iPLA_2$) activities (8). Therefore, experiments were conducted to determine whether ATP-stimulated PLD-2 might also involve PLA_2 activation in SMG acini. As expected, the cytosolic PLA_2 inhibitor ONO-RS-082 blocked the activation of PLD by ATP completely, suggesting a role for PLA_2 in the stimulation of PLD-2 by ATP in SMG acini (**Fig. 9**). Because the ONO-RS-082 inhibitor does not discriminate between $cPLA_2$ or $iPLA_2$ activities, experiments were carried out with the selective inhibitors, arachidonyl trifluoromethylketone ($AACOCF_3$) and (E)-6-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (bromoenol lactone) (BEL), which specifically block $cPLA_2$ and $iPLA_2$, respectively. Preincubation of the acini with optimum concentrations (100 μM) of either of these inhibitors (8) caused partial inhibitions of ATP-activated PLD, and the stimulation of this enzyme activity was inhibited further when the cells were preincubated with

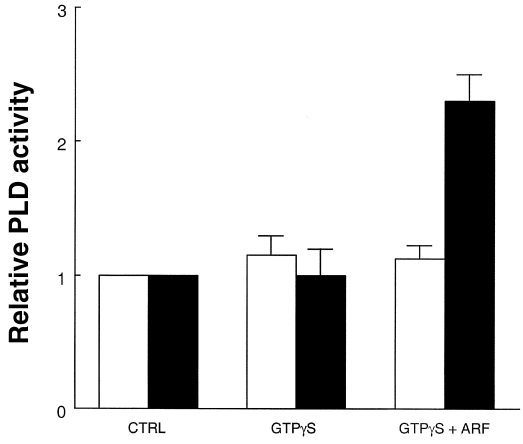

Fig. 7. Effect of recombinant ARF and GTPγS on PLD activity in membrane fractions of SMG ductal or acinar cells. SMG cells were labeled as indicated in Fig. 1. They were then permeabilized with 50 μg/ml of digitonin, and the membrane fractions from ducts or acini were separated from their cytosols according to Li et al. (20). Recombinant ARF1 (5 μM) and GTPγS (100 μM) were added to membrane fractions of acini (empty bars) or ducts (filled bars), as indicated. PLD activation was assayed over 20 min. Values are mean ± range of two independent experiments. In agreement with other work (20), recombinant ARF1 had no effect on basal PLD when added alone.

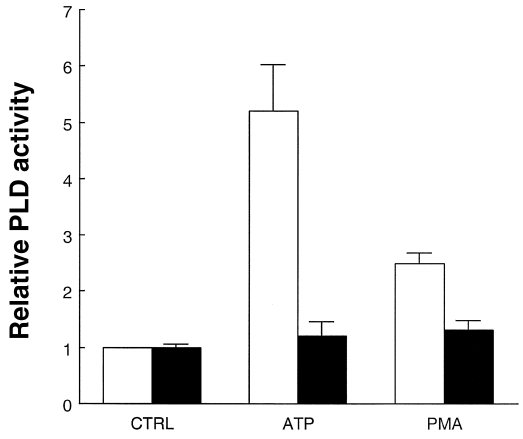

Fig. 8. Inhibition of ATP- or PMA-stimulated PLD by oleate in SMG acini. Cells were labeled and treated as indicated in Fig. 1, except that they were preincubated for 5 min in the absence (open bars) or in the presence (solid bars) of 1 mM oleate. Cells were then stimulated with 1.5 mM ATP or 100 nM PMA for 30 min, as indicated. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of ATP or PMA. Results are the mean ± SEM of three independent experiments.

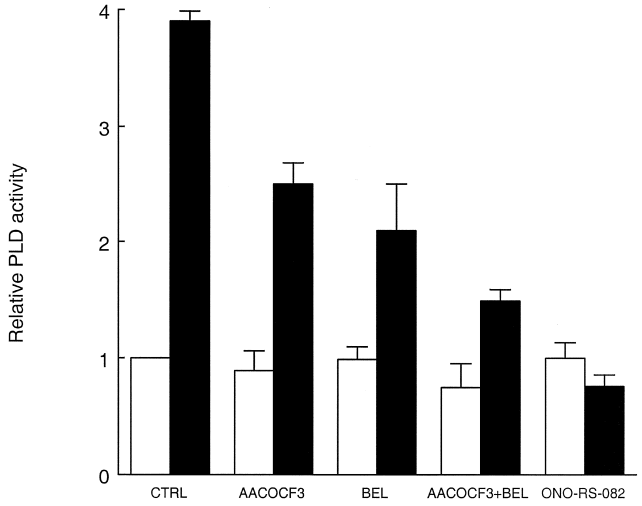

Fig. 9. Effect of the cytosolic PLA_2 inhibitors $AACOCF_3$, (E)-6-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (bromoenol lactone) (BEL) and ONO-RS-082 on ATP-stimulated PLD activity. SMG acini were labeled and treated as indicated in Fig. 1, except that they were preincubated for 5 min in the absence or in the presence of 100 μM of $AACOCF_3$ or BEL, or 500 μM of ONO-RS-082, as indicated. Cells were then treated with buffer (open bars) or stimulated with 1.5 mM ATP (solid bars) for 30 min. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of ATP. Results are the mean ± range of two independent experiments.

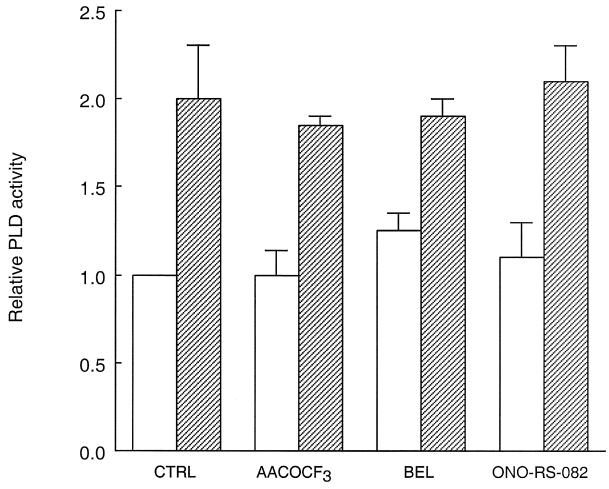

Fig. 10. Lack of an effect of the cytosolic PLA_2 inhibitors $AACOCF_3$, BEL, and ONO-RS-082 on carbachol-stimulated PLD activity in SMG ducts. Cells were labeled and treated as indicated in Fig. 1, except that they were preincubated for 5 min in the absence or in the presence of 100 μM of $AACOCF_3$ or BEL, or 500 μM of ONO-RS-082, as indicated. Cells were then treated without (open bars) or with 100 μM carbachol (hatched bars) for 30 min. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of ATP. Results are the means ± range of two independent experiments.

both PLA_2 inhibitors simultaneously (Fig. 9). To rule out a possible direct effect of these inhibitors on PLD, they were evaluated for specificity. This was accomplished by challenging SMG ductal cells with 100 μM carbachol, a drug which does not stimulate PLA_2 activity in this cell type (8), in the presence or absence of each of the PLA_2 inhibitors. **Figure 10** shows that carbachol activated PLD by about 2-fold, and this effect was not significantly altered by $AACOCF_3$, BEL, or ONO-RS-082. In addition, we found that 5 mM sulfasalazine, a drug used for treatment of inflammatory bowel disease, stimulated PLD by about 2.2 ± 0.1-fold (mean ± SEM of three independent experiments performed in duplicate) in Raw 264.7 macrophages in the absence of PLA_2 activation, and that this effect was not significantly changed by $AACOCF_3$, BEL, or ONO-RS-082. Taken together, these observations suggest an involvement of both $cPLA_2$ and $iPLA_2$ in the regulation of PLD-2 by ATP.

Effect of purinergic agonists and antagonists on the activation of PLD by ATP

To characterize the purinergic receptors coupled to the stimulation of PLD by ATP, the effects of several natural or synthetic agonists and antagonists were tested. It has been reported that synthetic Bz-ATP, an agonist that is considered to be selective for $P2Z/P2X_7$ purinergic receptors (51–53), is more potent than ATP in stimulating Ca^{2+} influx (21), arachidonic acid release (8), or kallikrein secretion in SMG cells (22). We now demonstrate that Bz-ATP, at relatively low concentrations (100 μM) potently stimulates PLD in SMG acini, (**Table 1**) whereas a similar concentration of ATP does not (Fig. 1, right panel). Agonists of other purinergic receptors were less effective or failed to activate PLD. In particular, and unlike ATP, 1.5 mM concentrations of the natural nucleotide triphosphates CTP, GTP, and UTP did not cause PLD stimulation (Table 1). Adenosine (1.5 mM) was also unable to activate PLD, and ADP (1.5 mM) was less potent than ATP or Bz-ATP in

TABLE 1. Effect of different purinergic agonists on PLD activity

	Relative PLD Activity
Control	1
CTP (1.5 mM)	1.29 ± 0.04
GTP (1.5 mM)	0.90 ± 0.01
UTP (1.5 mM)	0.68 ± 0.08
ADP (1.5 mM)	2.19 ± 0.34
Adenosin (1.5 mM)	1.20 ± 0.14
α,β-mATP (100 μM)	1.00 ± 0.19
Bz-ATP (100 μM)	3.72 ± 0.57
2MeSATP (100 μM)	1.31 ± 0.04
GTPγS (100 μM)	1.10 ± 0.13

Submandibular gland (SMG) acinar cells were labeled as in Fig. 1, and were then stimulated for 30 min with different purinergic agonists at the concentrations that are indicated in the presence of 1% ethanol. Phospholipase D (PLD) activity was measured by determining [^{3}H] phosphatidylethanol formation, as described in the Materials and Methods section. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared to that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of ATP. Results are the means ± SEM of three independent experiments.

stimulating this enzyme activity (Table 1). Other synthetic analogs, including α,β-methylene-ATP and the P2Y purinergic receptor agonist 2-methylthio-ATP (6), also failed to increase PLD activity in the acini (Table 1). The stimulation of PLD was potently inhibited by pretreatment of the acini with oxidized ATP (o-ATP) (**Fig. 11**, left panel), a selective P2Z/P2X$_7$ receptor antagonist (8, 54), or with suramin, a less-specific inhibitor than o-ATP but one which can also block P2Z/P2X$_7$ receptor activation (3) (Fig. 11, right panel). As expected, o-ATP also inhibited Bz-ATP-stimulated PLD (Fig. 11, left panel).

It is now recognized that the active form of ATP on P2Z/P2X$_7$ receptors is the free tetraionic form (ATP^{4-}) (1, 8, 55). Chelation of ATP by divalent cations might therefore decrease the concentration of the active form of ATP. It was previously demonstrated that free ATP^{4-} drops dramatically in the presence of 5 mM Mg^{2+} and loses its ability for activation of P2Z/P2X$_7$ receptors (8). Therefore, addition of Mg^{2+} into the incubation medium should also decrease PLD activation by ATP through P2Z/P2X$_7$ receptors. In agreement with this hypothesis, **Fig. 12** shows that increasing concentrations of Mg^{2+} block the activation of PLD by ATP. Figure 12 also shows that Ni^{2+} can also inhibit ATP-stimulated PLD potently. However, the mechanism whereby Ni^{2+} blocks PLD activation is rather distinct from that of Mg^{2+}, as Ni^{2+} ions do not decrease the concentration of ATP^{4-} (8). It has been suggested that Ni^{2+} can specifically affect the P2X component of purinergic responses by blocking Ca^{2+} uptake by the cells (6). Collectively, these results suggest an involvement of P2Z/P2X$_7$ receptors in the stimulation of PLD-2 by ATP. Because downregulation of PKC by PMA inhibited the activation of PLD by ATP (Fig. 4), it might be possible that PMA could cause a decrease in the expression of P2Z/P2X$_7$ receptors. In this regard, **Fig. 13** shows that PMA did not affect the expression of P2Z/P2X$_7$ receptors as assessed by RT-PCR.

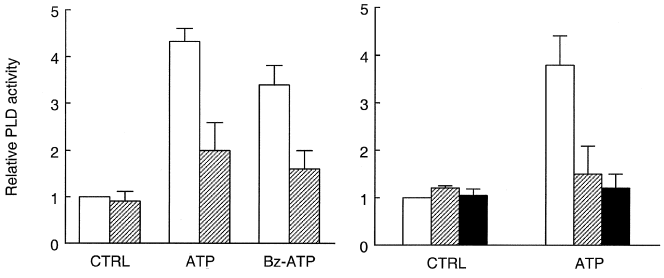

Fig. 11. Effect of the P2Z/P2X$_7$ receptor antagonists oxidized ATP (o-ATP) and suramin on the activation of PLD by ATP. SMG acini were labeled and treated as indicated in Fig. 1 except that they were preincubated for 60 min in the absence (open bars) or in the presence of 3 mM (hatched bars) of o-ATP (left panel), or for 3 min in the absence (open bars) or the presence of 3 mM (hatched bars) or 6 mM (solid bars) of suramin (right panel). Cells were washed once and then stimulated with 1.5 mM ATP for 30 min, as indicated. The results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of any addition. Results are the mean ± SEM of three independent experiments.

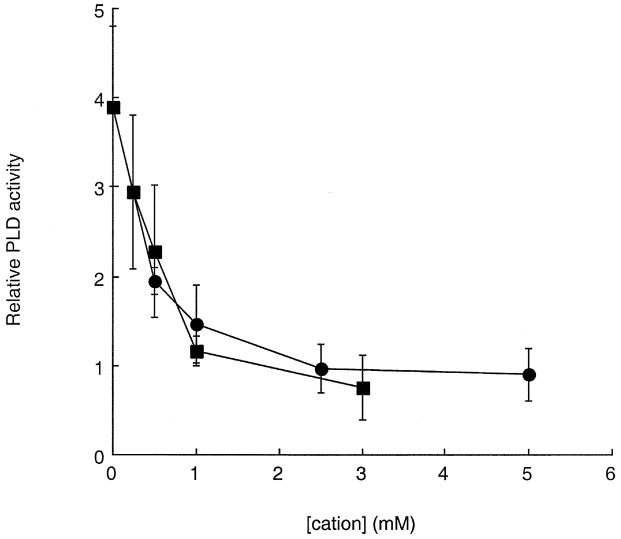

Fig. 12. Effect of various concentrations of Mg^{2+} or Ni^{2+} on the activation of PLD by ATP. Cells were labeled and treated as indicated in Fig. 1. Acini were then stimulated for 30 min with 1.5 mM ATP in the presence of increasing concentrations of Mg^{2+} (circles) or Ni^{2+} (squares). Values were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared with that in total lipids. Results are the mean ± SEM of three independent experiments, except for the points at 0.25 and 0.5 mM Ni^{2+}, where they are the mean ± range of two independent experiments.

DISCUSSION

ATP can elicit different biological actions depending on the concentration at which it is released. At relatively low concentrations (10–100 μM), ATP stimulates P2Y$_1$ receptors, leading to activation of PI-PLC (21, 22), whereas at higher concentrations (0.5–1.5 mM), it stimulates P2X

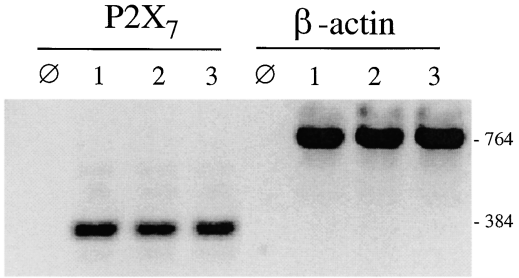

Fig. 13. RT-PCR detection of P2Z/P2X$_7$ mRNA in rat SMG acini in the absence or in the presence of PMA. Total RNA (2 μg) of SMG acini incubated in the absence (lane 1) or in the presence of 500 nM PMA for 4 h (lanes 2 and 3), or vehicle (ϕ) was subjected to RT-PCR, as described in Materials and Methods. RT-PCR amplifications of β-actin mRNA were performed in parallel in the same experiments as control to verify the integrity of the cDNA and the absence of contaminating gDNA. The numbering on the right corresponds to the size in base pairs of the amplicons. Data are representative of three independent experiments.

purinergic receptors and Ca^{2+} influx. Several reports have implicated Ca^{2+} in the stimulation of PLD in intact cells. This was concluded from experiments using Ca^{2+} ionophores or Ca^{2+} mobilizing agents on a variety of cell types (16, 41). However, the exact role of Ca^{2+} in agonist-stimulated PLD activation is not well understood. In fact, in cell homogenates or in permeabilized cell preparations, Ca^{2+} is not required for activation of PLD (3, 43). Also, the ability of P2Z/$P2X_7$ receptor agonists to stimulate PLD activity in the transformed murine macrophage cell line BAC1.2F5 was independent of Ca^{2+} ions (56). ATP (10–100 μM) stimulated IP_3 formation and intracellular Ca^{2+} mobilization in rat SMG acini, and removal of Ca^{2+} from the extracellular medium, or addition of EGTA, did not suppress this response to ATP (M. Fernández, E. Pérez-Andrés, and A. Marino, unpublished observations). Here, we show that low concentrations of ATP do not stimulate PLD, suggesting that generation of DAG and IP_3-mediated intracellular Ca^{2+} mobilization are not sufficient for PLD activation. These observations are in agreement with previous work on intact human lymphocytes (3), rat liver plasma membranes, broken human neutrophils, and permeabilized HL-60 cells [(3) and references therein], where concentrations of Ca^{2+} that approximated intracellular values (0.1–1 μM) failed to stimulate PLD. By contrast, at higher concentrations (0.5–1.5 mM), ATP stimulated PLD activity potently in the acini. Interestingly, these higher concentrations of ATP failed to stimulate the generation of IP_3 and the subsequent mobilization of intracellular Ca^{2+}. These observations indicate that PI-PLC may not be required for activation of PLD by ATP. However, in rat SMG acini, PLD activation by ATP was fully dependent on extracellular Ca^{2+}, as removal of this cation from the incubation medium, or addition of EGTA, completely blocked this effect. These observations are in agreement with those of Cabot's group, who showed that Ca^{2+} is not required for bradykinin-stimulated PLD activity in subcellular fractions of Madin-Darby canine kidney cells but is necessary for the effect of this agonist in intact cells (27).

PLD activity has also been shown to be regulated by sphingolipid metabolites. Although ceramides are potent inhibitors of PLD activity in a variety of cell types (13, 14, 18, 26, 28, 30–33), in human fibroblasts, ceramides or sphingosine can activate PLD (34). Our results indicate that it is unlikely that PLD activation by ATP is mediated by ceramide or its possible metabolism to sphingosine or sphingosine 1-phosphate, as ATP does not cause ceramide accumulation in the acini. Also, the cell-permeable [C_2]ceramide, exogenous sphingomyelinase, or sphingosine failed to stimulate PLD, and sphingosine 1-phosphate was a weak activator of this enzyme activity in the acini. In addition, treatment of the acini with [C_2]ceramide or exogenous SMase blocked PLD activation by ATP, further indicating that ceramides are negative signals for PLD activation, as previously demonstrated (18, 26, 28, 29, 33).

PKC plays a major role in the regulation of PLD, and in particular of the PLD-1 isozyme, through interaction with its N-terminus (16). As mentioned above, the involvement of PKC in ATP-stimulated PLD was evaluated by down-regulating PKC by prolonged treatment with phorbol esters, as they facilitate proteolysis of the enzyme at the plasma membrane of cells (57, 58), and by using selective PKC inhibitors. Although PKC isoforms have different susceptibility to proteolysis and to phorbol ester-induced down-regulation (57, 58), 4 h pretreatment of the acini with PMA rendered the acini insensitive to rapid stimulation by PMA, and the activation of PLD by ATP was substantially decreased. In addition, the selective PKC inhibitor chelerythrine inhibited ATP-stimulated PLD activity potently, further indicating that PKC is relevant for the activation of PLD by ATP in SMG acini.

A further objective of this work was to determine the specific PLD isoform that was stimulated by ATP in SMG acini. RT-PCR studies using primers specific for PLD-1 (*a* and *b*) or PLD-2 revealed that only PLD-2 was expressed in the acini. Our results also indicate that SMG acini lack the oleate-dependent PLD isoform. These observations are particularly important because the results that we have obtained from the mechanistic studies on the regulation of PLD by ATP can be entirely ascribed to PLD-2. Thus, we propose that PLD-2 is regulated by PKC and Ca^{2+} in intact cells, and that cytosolic PLA2 activation is an important factor in the stimulation of PLD-2 by ATP. This hypothesis is in agreement with that of Kim et al. (50), who have suggested a role for cytosolic PLA_2 in the regulation of PLD-2 in different leukocyte cell lines, whereas stimulation of PLD-1 was independent of PLA_2 activation. Also, although ATP can induce tyrosine phosphorylation of a variety of proteins, it is unlikely that this is a major mechanism for PLD-2 activation by ATP, as the tyrosine kinase inhibitor genistein had no effect on this process and the tyrosine phosphatase inhibitor vanadate did not enhance PLD activation by ATP. In support of this hypothesis, it has been suggested that tyrosine phosphorylation of PLD-2 is important for its interaction with SH_2-containing proteins but not for activity (16). In addition, our results indicate that PLD-2 is susceptible to inhibition by ceramides in vivo, and that it can be activated by sphingosine 1-phosphate.

The stimulation of PLD by ATP that we have observed can be mimicked by the selective P2Z/$P2X_7$ receptor agonist Bz-ATP, and is reduced by the selective antagonist o-ATP, and suramin. Furthermore, decreasing the concentration of ATP^{4-}, which is the active form of ATP that stimulates P2Z/$P2X_7$ receptors, with Mg^{2+} led to complete inhibition of PLD activation. Therefore, these observations suggest that PLD-2 stimulation by ATP in rat SMG acini may involve activation of ionotropic P2Z/$P2X_7$ receptors. However, the inhibitory effect of o-ATP on the ATP-stimulated PLD activity was incomplete, suggesting that purinergic receptors other than the P2Z/$P2X_7$ subclass might also be involved in this process. In this regard, recent work from our group using RT-PCR has demonstrated that $P2X_2$ and $P2X_4$ purinergic receptors are also expressed in rat SMG acini (M. Fernández, N. Chaïb, and A. Marino, unpublished observations). Also, it was reported recently that Bz-ATP might also stimulate $P2X_1$, $P2X_3$, or $P2X_4$ receptors (59, 60), suggesting that at least

some of the ATP-gated channels in SMG acini might be composed of a mixture of $P2X_7$ and other P2X subunits. Although Torres et al. (61) suggested that $P2X_7$ receptors are unlikely to form hetero-oligomeric structures, Lê et al. (5) have reported that the native ionotropic ATP receptors' diversity most likely arises from interactions between different P2X subunits, which generate heteromeric channels. It can thus be speculated that P2X receptors, other than $P2Z/P2X_7$, contribute to the activation of PLD-2 by extracellular ATP or Bz-ATP in SMG acini.

In conclusion, we show in this work that ATP stimulates PLD in rat SMG acini by a mechanism involving Ca^{2+} influx, PKC, and cytosolic PLA_2 activities, but that is independent of intracellular Ca^{2+} mobilization or PI-PLC activation. Most likely, the PLD isoform that is stimulated by ATP is PLD-2, as the acini do not express PLD-1 and lack oleate-dependent PLD activity. The results obtained with Bz-ATP and o-ATP indicate that PLD-2 activation by ATP may be mediated by the $P2Z/P2X_7$ receptor, which expression is not affected by PMA. However, an involvement of other ionotropic receptors in this process cannot be ruled out at present. Further work is required to elucidate whether ATP-stimulated PLD-2 is coupled to monomeric or to heteromultimeric P2X subunits in SMG cells.

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