Activation by P2X<sub>7</sub> Agonists of Two Phospholipases A<sub>2</sub> (PLA<sub>2</sub>) in Ductal Cells of Rat Submandibular Gland

COUPLING OF THE CALCIUM-INDEPENDENT PLA<sub>2</sub> WITH KALLIKREIN SECRETION*©

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Isolated ductal cells of rat submandibular gland phospholipid pools were labeled with [3H]arachidonic acid (AA). The tracer was incorporated preferentially to phosphatidylcholine (46% of the lipidic fraction). Extracellular ATP induced the release of [3H] AA to the extracellular medium in a time- and dose-dependent manner (EC<sub>50</sub> = 220 μM). Among other agents tested, only 2',3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (Bu-ATP) was able to mimic the effect of ATP (EC<sub>50</sub> = 15 μM), without activation of phospholipase C. The purinergic antagonists oxidized ATP, suramin, and Coomassie Blue partly inhibited the response to 1 mM ATP and 100 μM Bz-ATP; the response was also blocked by the addition of Mg<sup>2+</sup> or Ni<sup>2+</sup>. Expression of P2X<sub>7</sub> receptor mRNA in these cells was confirmed by reverse transcription-polymerase chain reaction. In the presence of extracellular calcium, the phospholipase A<sub>2</sub> inhibitor 2-(p-amylcinamoylamino)-4-chlorobenzoic acid (a nonspecific inhibitor), arachidonyl trifluoromethylketone (AACOCF<sub>3</sub>, an inhibitor of the calcium-dependent cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>)), and bromonolactone (an inhibitor of the calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>)) inhibited the release of [3H] AA induced by ATP and Bz-ATP. In the absence of extracellular calcium, the release of [3H] AA in response to the purinergic agonists was still observed; this response was not affected by AACOCF<sub>3</sub> and completely blocked by bromonolactone. ATP and Bz-ATP stimulated a calcium-independent secretion of kallikrein, which could be blocked by BEL but which was enhanced by AACOCF<sub>3</sub>. It is concluded that the P2X<sub>7</sub> receptor in ductal cells is coupled to kallikrein secretion through a calcium-dependent cPLA<sub>2</sub> and a calcium-independent iPLA<sub>2</sub>.

ATP plays an important role as an extracellular agonist that mediates its various effects by acting on specific membrane P2 receptor subtypes (1, 2). P2 receptors comprise receptors of the ligand-gated ion channel type, as well as of the G-protein-linked superfamily, termed P2X and P2Y, respectively (3). At present, several genes for P2X receptors have been cloned (4–8), but their physiological significance has not been fully established yet. One of the most recently cloned P2X receptors (the P2X<sub>7</sub> receptor) has a pharmacological profile typical of the receptor previously termed P2Z (9) with the photoactivatable analog of ATP, Bz-ATP, the most potent agonist. The P2Z receptor induces the formation of pores when exposed to concentrations of extracellular ATP in the 100 μM to 1 mM range (Refs. 10–12, but see also Ref. 13). In contrast to other P2X receptors, the P2X<sub>7</sub> receptor has a long COOH-terminal intracellular chain, which is by itself not responsible for the lytic properties of this receptor (14) but probably induces the formation of a second messenger involved in the lysis (12). In summary, the P2X<sub>7</sub>/P2Z-receptor share with the other P2X receptors the ability to open a non-selective channel and with P2Z receptors the induction of cell lysis by repeated applications of the agonist (8).

Since the pioneering work of Gallacher (15), ATP has been recognized as a major non-adrenergic non-cholinergic stimulus of saliva secretion. Salivation, like other exocrine secretions, occurs in two steps; (a) acinar cells secrete an isotonic plasmalike fluid, and (b) the electrolyte composition of this primary secretion is modified during its transfer to the mouth by the ductal tree (16). The ducts reabsorb Na<sup>+</sup> and Cl<sup>-</sup> and secrete K<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> (17). The study of these two phases of the secretory process has been facilitated by the description of an improved technique to separate ducts and acini (18). It could be observed that extracellular ATP increased the intracellular

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concentration of calcium ([Ca^{2+}]) both in rat submandibular gland (RSMG) acini and ducts. This result suggested that ATP might regulate both phases of secretion. In acini, ATP increased the intracellular concentration of Na⁺ (19), activated the Na⁺/H⁺ exchanger, and opened a chloride channel (20). It also inhibited the response to agonists activating the 1,0-phosphatidylglycerol, 4,5-bisphosphate-selective phospholipase C (21). Two purinergic responses were reported in pure RSMG ducts. At low concentrations, ATP activated a P2Y₁ receptor, while at high concentrations, it stimulated a P2X receptor (18, 22). The ionotropic receptor triggered the secretion of kalikrein. The molecular characterization of the ionotropic receptor present on RSMG ductal cells has not been achieved yet. Both P2X₄ and P2X₇ transcripts have been observed in rat submandibular glands (23) and, according to Buell et al. (6), P2X₄ are expressed in RSMG acini but not in RSMG ducts. It can thus be speculated that the P2X receptors expressed in ducts are of the P2X₇ type.

The functional consequences of the activation of this receptor have not been determined. The coupling of some P2Y receptors to phospholipases C and phospholipases A₂ has been clearly established (24–26). Reports on the regulation of the activity of phospholipases by P2X receptors are more scarce (27–29). The purpose of this work was to study the contribution of ionotropic purinergic receptors to the release of arachidonic acid by RSMG ductal cells. The phospholipases A₂ involved in this response and their role in the secretion in response to purines were also explored.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fura-2-acetoxymethyl ester (AM) was from Molecular Probes (Eugene, OR). 2-Methylthioadenosine 5’-triphosphate (2-MeSATP) was purchased from ICN Biochemicals (Costa Mesa, CA). Collagenase P, bovine serum albumin (BSA, fraction V), Coomassie Brilliant Blue, adenosine 5’-triphosphate (ATP, sodium salt), and ATP-γ-S were from Boehringer Mannheim (Mannheim, Germany). 2,3-Dialdehyde hydride ATP (periodate-oxidized ATP), Bz-ATP, thapsigargin, A23187, diethylentriaminepentaacetic acid (DTPA), Na₂-benzoyl-orn-arginine p-nitroanilide, HEPEs, EGTA, DNase I (from bovine pancreas), dextran, carbamylcholine chloride, epinephrine, isoproterenol, ionomycin, and the pH was adjusted to 7.4 with NaOH. Ten minutes after the addition of an incubation mixture, radioactivity of the samples was measured in a scintillation spectrometer (model 2000 CA, TRI-Carb, Packard). In order to estimate the radioactivity already present in the medium at the start of each assay, several samples of the cellular suspension were taken during the experiments, mixed with 400 μl of HBS, and directly centrifuged, and the radioactivity present in the supernatant was counted. The specific activities were used to calculate the values and standard error and then the radioactivity of the supernatant was expressed as disintegrations per minute (dpm) per 10⁶ cells.

**Isolation of RSMG Ducts—**Male Sprague-Dawley rats (200 g) fed ad libitum and given free access to water were used. The rats were killed by exposure to diethylether, and the glands were immediately dissected and finely minced. The suspension of ductal cells was prepared as described previously (18) with some modifications. Briefly, the minced glands of one rat were digested in the presence of 2.6 mg of collagenase F (1.52 units/ml) for 20 min at 37 °C under constant shaking (90 cycles/min) in 10 ml of HEPEs-buffered saline (HBS) containing (mM): 24.5 HEPS, 96 NaCl, 6 KCl, 2.5 NaH₂PO₄, 11.5 glucose, 5 pyruvate, 5 glutamate, 5 fumarate, 1% (w/v) glucose-free amino acid mixture (Life Technologies, Inc.), and 0.125% (w/v) bovine serum albumin (BSA). The pH was adjusted to 7.4 with NaOH. Ten minutes after the beginning of the digestion and at the end of the digestion, the cells were aspirated several times with 10-, 5-, and 2-ml glass pipettes. The suspension was then washed three times with an isotonic saline solution, resuspended in 10 ml of collagenase-free HBS and incubated for 10 min in the presence of 0.06 mg of DNase I (420 units/mg). The crude suspension was pipetted again, filtered through a nylon mesh and passed through a 25-gauge needle 5 times in a saline solution. Cells were resuspended in 4 ml of HBS and distributed in 4 ml of Percoll (1.077 g/ml, Pharmacia) and 1% bovine serum albumin (BSA) in 4 ml of Percoll solution (40%). The tubes were centrifuged at 4,000 × g at 4 °C for 10 min. At the end of the centrifugation, one population of cells had sedimented while the other had remained on the top of the Percoll. The microscopic examination revealed that the upper band was a ductal suspension while acini had sedimented through the Percoll. The protein concentration was 3.6 ± 0.6 mg/ml and the radioactivity of the upper layer was enriched. It was further purified by a Bio-Rad protein assay kit using γ-globulin as standard. The assay of kallikrein (a ductal marker) confirmed that the upper layer was already 4.4-fold in kallikrein activity with respect to the crude cellular suspension. Ducts were aspirated, washed, and resuspended in HBS buffer for further experiments.

**Isolation of [³H]Arachidonic Acid, [methyl-¹⁴C]Choline and myo-[²-³H]inositol to Ductal Cells—**The ducts from two glands of one rat were resuspended in 1 ml of HBS in the presence of 0.5 mM CaCl₂, 3 μCi/ml [³H]Arachidonic acid ([³H]AA) were added, and the suspension was incubated at 25 °C with gentle shaking. At various times, 50-μl aliquots were transferred to Eppendorf tubes and centrifuged at 16,000 × g for 1 min. The pellet was washed with 0.9% NaCl, the lipids were extracted, and the incorporation of [³H]AA into the lipid fraction was determined by TLC analysis. The upper phase of the lipid fraction was evaporated to dryness and the sample was dissolved in 6 ml of chloroform:methanol (2:1). These results were used as blank values and used to estimate the radioactivity already present in the medium at the start of the isotopic labeling, ductal cells were washed and incubated for 1 h in 6 ml of calcium-free HBS in the absence of the tracer. After this incubation, the ducts were washed again and resuspended in 6 ml of calcium-free HBS. Assays were performed at 37 °C under constant shaking and started by adding 100 μl of cell suspension to 400 μl of HBS containing CaCl₂ (final concentration 0.5 mM) and the agonists. The reaction was stopped by centrifugation for 30 s at 10,000 × g. Four hundred μl of the upper phase were added to the 900 μl of scintillation mixture. Radioactivity of the samples was measured in a scintillation spectrometer (model 2000 CA, TRI-Carb, Packard). To study the metabolism of phosphatidylcholine and phosphatidylserine, ductal cells from 1 or 3 rats, respectively, were incubated for 90 min as described above in 1 ml of HBS containing 0.5 mM CaCl₂ and 4 μCi/ml [methyl-¹⁴C]choline or 25 μCi/ml myo-[²-³H]inositol. Assay for [³H]Arachidonic Acid Release—At the end of the isotopic labeling, ductal cells were washed and incubated for 1 h in 6 ml of calcium-free HBS in the absence of the tracer. After this incubation, the ducts were washed again and resuspended in 6 ml of calcium-free HBS. Assays were performed at 37 °C under constant shaking and started by adding 100 μl of cell suspension to 400 μl of HBS containing CaCl₂ (final concentration 0.5 mM) and the agonists. The reaction was stopped by centrifugation for 30 s at 10,000 × g. Four hundred μl of the upper phase were added to the 900 μl of scintillation mixture. Radioactivity of the samples was measured in a scintillation spectrometer (model 2000 CA, TRI-Carb, Packard). In order to estimate the radioactivity already present in the medium at the start of each assay, several samples of the cellular suspension were taken during the experiments, mixed with 400 μl of HBS, and directly centrifuged, and the radioactivity present in the supernatant was counted. The specific activities were used to calculate the values and standard error and then the radioactivity of the supernatant was expressed as dpm/mg protein.

**Isolation of RSMG Acini—**Acini were isolated from rat submandibular glands (RSMG) of one pair of glands of one rat. The paired glands were homogenized in 4 ml of 0.9% NaCl and transferred to a 20 ml of chloroform:methanol:HCl (200:100:1). After 30 min in an ice bath, a final concentration of 0.1 mM HCl was added and the tubes were vortexed and let at room temperature. The tubes were then centrifuged at 200 × g for 15 min; 200 μl of the organic phase were vacuum-dried in an automatic SpeedVac concentrator (Savant AS290) and resuspended in 40 μl chloroform. Aliquots (30 μl) were spotted on 20 × 20-cm Silica Gel
60 F_{254} plates. An ascending chromatography was performed using chloroform:methanol:acetic acid:water (75:45:12:3) as the eluant for the analysis of phospholipids. Spots were identified by comigration with authentic standards, which were visualized by exposure to iodine. The silica gel containing radioactivity was scraped into scintillation vials and the supernatants were pooled. The fractions were analyzed using the GeNetics Computer Group program GCG Primer. Upstream and downstream primers were based on unique sequences within exon 8 and exon 12, respectively, stretching bases 958–980 (P2X7.up) and 30210–30237 (P2X7.down) of the P2X7 sequence (GenBank accession no. X95682). The amplified product was then predicted to be 384 base pairs in length. These regions correspond to the intracelular and carboxy-terminal extracellular loops, respectively, of the corresponding amino acid sequence. PCR was performed as described previously (32) with some modifications. A hot-start touchdown PCR protocol with thinned-walled tubes was employed involving denaturation at 95 °C and extension at 72 °C for every two cycles until 56 °C, at which all subsequent annealings were performed. Each step lasted for 50 s, and a total of 45 cycles were performed. The reaction mixture contained 1.5 mM magnesium. Amplified products were analyzed by electrophoresis in 1.8% agarose gels and viewed with ethidium bromide staining. A X174 HaeIII digest was used as a size standard. In order to confirm that the amplicons observed corresponded to RNA and not contaminating genomic DNA, we performed PCR under identical conditions with rat genomic DNA. The amplicon in this case was >3000 base pairs, indicating the presence of the four predicted introns in the gDNA between exons 8 and 12 (data not shown). Thus, the PCR products obtained from the submandibular gland and from the acinar and ductal cells were derived from expressed mRNA and not genomic DNA.

Calcium Measurements—Calcium concentration was determined as described previously (18). Briefly, ducts from one rat were resuspended in 3 ml of HBS. Aliquots (1 ml) were incubated at 25 °C with 1 ml of HBS buffer in the presence of 0.5% (w/v) BSA, 0.25 mM CaCl{\textsubscript{2}} and 2 mM Fura-2/AM. After 45 min, 1 ml of the suspension was removed, washed with isotonic NaCl, and resuspended in 2 ml of magnesium-free HBS without BSA or amiloride. The cells were constantly stirred in the cuvette, and the excitation wavelength was switched every second from 340 nm to 380 nm (slit width 4 nm). The light emitted at 505 nm was recorded (slit width 8 nm). The voltage of the photomultiplier was 700–750 V. At the start of each assay, the signal observed after excitation at 345 nm was arbitrarily set at 50% maximal scale by adjusting this voltage. At the end of the assay, the traces were calibrated with the successive addition of 0.1% (v/v) digitonin and 40 mM EGTA (pH 8.5 with Tris). Autofluorescence was measured at both wavelengths in cells that were not loaded with the Ca\textsuperscript{2+} indicator and was subtracted from all the data before calculation of the ratios. The calcium concentration was evaluated by the ratio method (33), using a K\textsubscript{D} value of 263 nM.

Activity of the Polyphosphoinositide-specific Phospholipase C—The ductal cells obtained from 3 rats were labeled with nycodazodium free of magnesium as described previously (21). At the end of the incubation, the cells were washed twice with 10 ml of HBS in the absence of labeled inositol and finally resuspended in 4.5 ml of HBS in the presence of 10 mM LiCl. They were preincubated in this medium for 10 min at 37 °C. Aliquots (100 μl) were incubated in a final 500 μl of HBS medium in the presence of the tested agents for 5 min at 37 °C. At the end of the incubation, the samples were centrifuged at 10,000 × g for 30 s. The supernatant was discarded, and 500 μl of 10% (w/v) ice-cold trichloroacetic acid were added to the pellet. The samples were centrifuged for 2 min at 10,000 × g, and the supernatant was transferred to glass tubes. The pellets were washed again with 500 μl of 10% trichloroacetic acid, centrifuged and the supernatants were pooled. The trichloroacetic acid was extracted with diethyl ether saturated with water, neutralized with 1.9 M KOH and diluted with 8 ml of 10 mM HEPES, 2 mM EDTA (pH 7.4). The inositol phosphates were isolated using a Dowex AG1-X8 column (34). The glycerophosphoinositol was eluted with 12 ml of 30 mM ammonium formate. The inositol derivatives (inositol mono-, bis-, and trisphosphates) were eluted simultaneously from the Dowex column using 12 ml of 0.7 mM ammonium formate and 0.1 mM formic acid. The radioactivity eluted off the column was quantified by liquid scintillation counting. Each determination was performed in triplicate.

Kallikrein Secretion—The ductal cells isolated from 1 rat were re-suspended in 4 ml of magnesium-free HBS. The stimulation of the cells with the agonist was performed for 10 min as described for the [3H]AA release assay. At the end of the incubation, the cells were centrifuged for 30 s at 10,000 × g. Four hundred μl of the supernatant were transferred to Eppendorf tubes and kept on ice. The kallikrein assay was performed as described previously (18). The assay was run in a 1-ml plastic cuvette using 300 μl of Tris buffer (0.5 M, pH 8.2), 300 μl of substrate solution (2 mg/ml N,N-benzoyl-L-arginine p-nitroanilide), and 100 μl of the sample. The absorbance was measured at 405 nm during 3 min in a Uvikon 943 spectrophotometer. The results were plotted as absorbance versus time, and the activity of the enzyme was calculated using the slope of the linear part of the curve. An aliquot of the ductal suspension was homogenized by sonication (2 pulses of 5 s at 8 μm with a Soniprep 150) and assayed for kallikrein content. This result gave an estimate of the total content of kallikrein in the cell suspension. Results were expressed as a percentage of the total kallikrein content released during the incubation at 37 °C. These results were corrected with regard to the kallikrein present in the medium prior to the incubation of the cells with the agonists.

Statistical Analysis—Unless otherwise indicated, values in figures are given as means ± standard error (S.E.) of n experiments performed in n different cell preparations. Statistical significance of the results was determined using a Student’s t-test and represented by * (p < 0.05), ** (0.001 < p ≤ 0.01), # (0.01 < p ≤ 0.05), and NS (not significant) (0.05 ≤ p).

RESULTS

Release of [3H]AA from RSMG Ductal Cells in Response to Purinergic Agonists—RSMG ductal cells were prelabeled with [3H]AA and incubated for various times in the presence of either 1 mM ATP or 100 μM Bz-ATP. As shown in Fig. 1, the two purinergic agonists increased the release of [3H]AA from prelabeled cells. The maximum stimulation was observed after 15 min. After 20 min of exposure to ATP or Bz-ATP, the release of [3H]AA was increased by 216 ± 13% and 310 ± 12%, respectively. The other purinergic agonists tested (2-MeSATP, AMP-PNP, α,β-methylene ATP, β,γ-methylene ATP, 5′-adenosine tetraphosphate, ATPγS, ADP, and UTP) were unable to elicit a significant response in terms of [3H]AA release (Table I). Among the nonpurinergic secretagogues tested, only 100 μM carbachol was able to induce a small effect (118 ± 8%). The stimulation of the cells for 20 min with calcium ionophores (100 nM ionomycin or 1 μM A23187) or the depletion of the intracellular calcium stores in cells incubated for 20 min with 1 μM thapsigargin had no significant effect on the release of [3H]AA from the cells (Table I). In separate experiments, it was shown that all these agents (epinephrine, carbachol, ionomycin, and thapsigargin) increased the [Ca\textsuperscript{2+}] in the ductal suspension (data not shown).

The release of [3H]AA in response to ATP and Bz-ATP was dose dependent (Fig. 2). ATP significantly increased the release of [3H]AA at 100 μM, its half-maximal and maximal effects being observed at 220 μM and 1 μM, respectively. The RSMG ductal cells were more sensitive to Bz-ATP, which significantly increased the release of [3H]AA at concentrations as low as 10 μM. The EC\textsubscript{50} and the maximal concentration for Bz-ATP were 15 and 100 μM.

Effect of Purinergic Inhibitors and Antagonists on the Release of [3H]AA—To further characterize the purinergic receptors coupled with the release of [3H]AA, the effect of several antagonists was tested (Table II). Among these antagonists, ATP (aATP) is an inhibitor of P2\textsubscript{x} receptor in human lymphocytes (29) and in mouse macrophages (35). The cells were preincubated with aATP for 60 min before exposure to the purinergic agonist. By itself, aATP did not affect the basal release of [3H]AA. At a 100 μM concentration, aATP inhibited by 52 ± 6% and 46 ± 6% the [3H]AA release in response to ATP and Bz-ATP.
blocks the response of submandibular acini to Bz-ATP (21). At
inhibitor tested. It has been reported that Coomassie Blue
iments performed in triplicate.

100 μM Bz-ATP, respectively. The decreases observed on cellular
[3H]AA-labeled phospholipids in Bz-ATP-stimulated cells at 20
min were also partially inhibited by 10 μM Coomassie Blue
(data not shown). These observations indicated that the [3H]AA
release from the stimulated cells could be at least in part
secondarily coupled to the activation of P2Y receptor.

Effect of Divalent Cations on the Release of [3H]AA in Response to Purinergic Agonists—It is generally recognized that the
active form of ATP on P2X7/P2Y receptors is the free
tetraionic form (ATP4−). The complexation of ATP by divalent
cations might thus decrease the concentration of the active
form. However, divalent cations might also block the nonspeciﬁc
cation channels coupled to the ionotropic receptors. Magnesium
(18) and nickel (36) were used in these studies. The
concentrations of the different forms of ATP in the presence of
either Ni2+ or 5 mM Mg2+ was estimated using the Solgsawater
program (37) and the equilibrium constants from different well
documented sources (38, 39). According to our calculations
(Table III) in a Ni2+ or Mg2+ free medium containing 1 mM ATP

\[ [\text{ATP}^{4-}] = \frac{[\text{ATP}^{4-}]_{\text{free}}}{K_{\text{diss}}^{	ext{Ni2+}}} \]

\[ [\text{ATP}^{4-}] = \frac{[\text{ATP}^{4-}]_{\text{free}}}{K_{\text{diss}}^{	ext{Mg2+}}} \]

where \([\text{ATP}^{4-}]_{\text{free}}\) is the free concentration of ATP4−, and KdissNi2+ and KdissMg2+ are the dissociation constants for Ni2+ and Mg2+, respectively. The calculations were performed using the Solgsawater program (37) and the equilibrium constants from different well-documented sources (38, 39). The results indicate that the presence of Ni2+ or Mg2+ significantly decreases the free concentration of ATP4−, which likely affects the activity of P2X7/P2Y receptors.

\[ [\text{ATP}^{4-}]_{\text{free}} = \frac{[\text{ATP}]}{K_{\text{diss}}^{	ext{Ni2+}}} \]

\[ [\text{ATP}^{4-}]_{\text{free}} = \frac{[\text{ATP}]}{K_{\text{diss}}^{	ext{Mg2+}}} \]

where [ATP] is the total concentration of ATP, and KdissNi2+ and KdissMg2+ are the dissociation constants for Ni2+ and Mg2+, respectively.}

\[ R = \frac{[\text{ATP}]_{\text{free}}}{[\text{ATP}]_{\text{total}}} \]

\[ R = \frac{[\text{ATP}^{4-}]_{\text{free}}}{[\text{ATP}^{4-}]_{\text{total}}} \]

where R is the relative release of [3H]AA, [ATP]_{\text{free}} is the free concentration of ATP, and [ATP]_{\text{total}} is the total concentration of ATP. The calculations were performed using the Solgsawater program (37) and the equilibrium constants from different well-documented sources (38, 39). The results indicate that the presence of Ni2+ or Mg2+ significantly decreases the relative release of [3H]AA, which likely affects the activity of P2X7/P2Y receptors.

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and 0.5 mM Ca\(^{2+}\), the ATP\(^{4-}\) concentration was 29.7 \(\mu\)M. The ATP\(^{4-}\) concentration was barely affected by the presence of different concentrations of Ni\(^{2+}\). It was calculated that the addition of 1 mM Ni\(^{2+}\) should decrease the [ATP\(^{4-}\)] to 25.8 \(\mu\)M, a concentration able to activate the P2Z receptors of rat parotid gland (40). In the presence of 5 mM Mg\(^{2+}\), the free ATP\(^{4-}\) drops dramatically to 7.4 \(\mu\)M. At this concentration, ATP is not able to stimulate the low affinity receptor described previously (18).

Five mM MgCl\(_2\) inhibited the release of \([^{3}H]AA\) in response to 100 \(\mu\)M Bz-ATP and 1 mM ATP by 95 \(\pm\) 4\% and 91 \(\pm\) 6\%, respectively. It can be concluded that ATP\(^{4-}\) is the form of ATP promoting the release of \([^{3}H]AA\) from cellular phospholipids. The release of \([^{3}H]AA\) in response to Bz-ATP and ATP was also inhibited when various concentrations of Ni\(^{2+}\) were added to the medium (Fig. 3). The half-maximal inhibitory concentrations were 0.18 and 0.24 mM with respect to 100 \(\mu\)M Bz-ATP and 1 mM ATP. The response to the purinergic agonists was totally abolished in the presence of concentrations of Ni\(^{2+}\) higher than 0.75 mM. The inhibition exerted by 0.75 mM Ni\(^{2+}\) was demonstrated by RT-PCR using rat whole brain as a positive control. As shown in Fig. 5, P2X7 transcripts were detected in the whole RSMG as well as in both acinar and ductal purified fractions. The expression of the P2X7-receptor mRNA in ducts and acini of the rat submandibular gland (8) and termed P2X7 according to the current classification. This hypothesis was tested in parallel experiments, where the effect of nickel on the [Ca\(^{2+}\)] in response to purines was tested. RSMG ductal cells were loaded with Fura-2 and resuspended in HBS medium in the presence of 1 mM CaCl\(_2\). When the cells were incubated in control conditions, the addition of 100 \(\mu\)M Bz-ATP to the medium sharply increased the [Ca\(^{2+}\)] from 202 \(\pm\) 41 to 646 \(\pm\) 94 nM (n = 3, p < 0.01) and the [Ca\(^{2+}\)], continued to rise slowly thereafter (Fig. 4, right panel). The addition of 1.5 mM DTPA at 10 min caused a slight drop on the calcium level (from 1264 \(\pm\) 42 to 1067 \(\pm\) 112 nM, 0.05 < p). This decrease might be due to the binding of some calcium to DTPA and the subsequent decrease of the free calcium concentration in the medium. When the RSMG ductal cells were resuspended in the presence of 0.75 mM Ni\(^{2+}\), the initial response to 100 \(\mu\)M Bz-ATP was completely inhibited (Fig. 4, right panel). The [Ca\(^{2+}\)], increased slowly reaching 367 \(\pm\) 95 nM at 10 min. It can also be noted that the intracellular Fura-2 was not quenched by nickel, confirming that at the opposite to calcium and magnesium, this divalent cation cannot enter the RSMG ductal cells via the nonspecific cation channels coupled to the P2X7 receptors. The chelator added 10 min after Bz-ATP almost totally reversed, for the first 10 s, the inhibition exerted by Ni\(^{2+}\) on the response to Bz-ATP. Five minutes after the addition of DTPA, the [Ca\(^{2+}\)], increased to 550 nM.

Expression of P2X7 Receptor mRNA in RSMG Cells—The P\(_{\text{zz}}\) receptor has been recently cloned in rat brain by Surprenant et al. (8) and termed P2X7, according to the current classification. The expression of the P2X7-receptor mRNA in ducts and acini was demonstrated by RT-PCR using rat whole brain as a positive control. As shown in Fig. 5, P2X7 transcripts were detected in the whole RSMG as well as in both acinar and ductal purified fractions.

Origin of the \([^{3}H]AA\) Released in Response to ATP and Bz-ATP—As stated under “Experimental Procedures,” \([^{3}H]AA\) was preferentially incorporated into phosphatidylcholine (PC), which represented 46\% of the total lipid-labeled fraction, whereas the incorporation into phosphatidylethanolamine (PE) and phosphatidylserine + phosphatidyl inositol (PS+PI) was 18.8\% and 5\%, respectively. When \([^{3}H]AA\)-labeled cells were

### Table III

| [ATP species] | HBS | \([\text{Mg}^{2+}] (5 \text{ mM})\) | \([\text{Ni}^{2+}] (1 \text{ mM})\) |
|--------------|-----|-----------------|-----------------|
| \(\text{ATP}^{4-}\) | 29.7 | 7.4 | 28.3 |
| \(\text{ATPNa}^{3-}\) | 810 | 203 | 772 |
| \(\text{ATPCa}^{2+}\) | 84.5 | 26.9 | 81.5 |
| \(\text{ATPCa}_{2}\) | 32.5 | 13.2 | 31.7 |
| \(\text{ATP}^{K}\) | 39.6 | 9.93 | 37.7 |
| \(\text{ATPH}\) | 3.49 | 0.87 | 3.32 |
| \(\text{ATPMg}^{2+}\) | 0 | 594 | 0 |
| \(\text{ATPMg}_{2}\) | 0 | 144 | 0 |
| \(\text{ATPNi}^{2+}\) | 0 | 45.5 | 77.1 |
| \(\text{ATPNi}_{2}\) | 0 | 0 | 0.00131 |

The concentration of different species of complexed ATP (in \(\mu\)M) has been calculated with the computer program Solgaswater, considering all the possible forms in a medium containing 1 mM ATP (total concentration) and 24.5 mM HEPES, 0.5 mM CaCl\(_2\), 9.6 mM NaCl, 6 mM KCl, 2.5 mM Na\(_{2}\)PO\(_4\), 11.5 mM glucose, 5 mM pyruvate, 5 mM glutamate, 5 mM fumarate, pH = 7.4, in the presence of or absence of 5 mM Mg\(^{2+}\) and different concentrations of Ni\(^{2+}\). For clarity of the table, minor species (<1 \(\mu\)M) have been omitted.
stimulated with 1 mM ATP or 100 μM Bz-ATP, we observed at 10 and 20 min a 22% and 32% decrease into the content of [3H]AA-labeled PC pool, respectively (Table IV). At 20 min a significant 23% decrease of the [3H]AA-labeled PE pool was also observed in the ATP-stimulated cells. The response to ATP was completely blocked by the addition of 5 mM MgCl2 to the medium (data not shown), confirming the contribution of P2X7 receptors in this response. The PS+Pi [3H]AA-labeled pool was not significantly affected by ATP or Bz-ATP (data not shown).

No significant variations of the [3H]AA-labeled phosphatidic acid and diacylglycerol pools in response to ATP or Bz-ATP could be observed and the amount of dpm lost by the phospholipid fraction correlated well with the radioactivity released in the medium. Thus, the radioactivity lost from the total phospholipid fraction in Bz-ATP-stimulated ducts with respect to controls at 10 and 20 min was 2038 ± 165 and 4036 ± 456 dpm, respectively. By comparison, the radioactivity found in the supernatants in the same conditions was 1865 ± 889 and 4883 ± 156 dpm. The effect of purinergic agonists on the metabolism of PC was further characterized using [14C]choline-labeled cells. One hundred μM Bz-ATP time-dependently decreased the content of [14C]choline-labeled PC with a maximal 33 ± 3% decrease observed after 20 min (data not shown), a value similar to the value observed with [3H]AA-labeled PC. Bz-ATP provided most of the [3H]AA released in response to purinergic agonists, suggesting that a phospholipase A2 could be activated by P2X7 agonists in RSMG ductal cells either directly or after the activation of phospholipase D. The purpose of the next experiments was to further characterize the mechanisms involved in the release of [3H]AA from the ATP-stimulated RSMG ductal cells.

**Contribution of Various Phospholipases A2 on the Release of [3H]AA**—Various inhibitors were used to study the implication of a phospholipase A2 in the release of [3H]AA from RSMG ductal cells. Cells were preincubated for 5 min with ONO-RS-082 (a nonspecific inhibitor of PLA2 since it is able to inhibit both the cytosolic and the secreted forms of the enzyme; Ref. 41) before exposure to either 1 mM ATP or 100 μM Bz-ATP. Five hundred μM ONO-RS-082 fully inhibited the response to the two agonists (IC50 67 and 85 μM for the response to ATP and Bz-ATP, respectively. In the absence of agonists, ONO-RS-082 had no significant effect on the release of [3H]AA. Two other more specific inhibitors were tested next. AACOCF3 is a potent, slow, tight-binding, reversible inhibitor of cPLA2 and it proved to be highly specific for the 85-kDa cytosolic calcium-dependent PLA2 (cPLA2) (42). In our conditions, incubation of the cells with AACOCF3 for 2 min prior to the addition of the purinergic agonists produced a concentration-dependent decrease in the stimulated [3H]AA release (Fig. 6, left panel), whereas it had no effect on the basal [3H]AA release. The inhibition averaged 15 ± 3% at a 300 nM AACOCF3 concentrations. Half-maximal inhibition could be observed at 2 μM and at a maximal 100 μM concentration, AACOCF3 inhibited by 84 ± 4% the response to 1 mM ATP (data not shown) and by 73 ± 6% the response to 100 μM Bz-ATP (Fig. 6, left panel). A full inhibition could never be observed. These results suggested that a PLA2 was at least partly responsible for the release of [3H]AA in response to purinergic agonists but that some other PLA2 might also be involved. We examined the possibility that a calcium-independent PLA2 was present in RSMG ducts. The release of [3H]AA was measured in the absence of calcium but in the presence of 0.5 mM EGTA. In these conditions, Bz-ATP could not increase the [Ca2+]i (18, data not shown). The release of [3H]AA in response to Bz-ATP was inhibited by 66 ± 3% but the stimulatory effect of Bz-ATP on the release of [3H]AA remained highly significant when compared with control (p < 0.001). The addition of various concentrations of AACOCF3 to the medium did not affect this calcium-insensitive release of [3H]AA in response to Bz-ATP (Fig. 6, left panel). This result confirmed that ACOOF3 inhibited the cPLA2, which is a calcium-dependent PLA2. The presence of a calcium-insensitive (iPLA2) in RSMG ducts was confirmed with BEL, an inhibitor of the iPLA2 (43). In the presence of extracellular calcium, BEL slightly inhibited the basal release of [3H]AA by 18% and 11% at 3 and 10 μM, respectively, whereas it had no significant effect at 30 and 100 μM. BEL dose-dependently inhibited the release of [3H]AA in response to either ATP (IC50 1 μM; data not shown) or Bz-ATP (IC50 2 μM; Fig. 6, right panel). At a maximal 100 μM concentration of BEL, the release to the two purinergic agonists was inhibited by 71 ± 3%.
were preincubated for 2 min with various concentrations of AACOCF3. AACOCF3 decreased by, respectively, 90 % the radioactivity present in the bands of PC, PE, and PS+Pi. Data represent mean ± S.E. of three independent experiments performed in triplicate. The percentage of 3H content with respect to unstimulated controls and statistical significance are also shown.

| Time | PC | % | PE | % | PS+Pi | % |
|------|----|---|----|---|-------|---|
| 0    | 11,843 ± 1563 | 100 | 4773 ± 605 | 100 | 1513 ± 644 | 100 |
| 10 min | 9366 ± 604 | 78 ± 7 (*) | 4445 ± 195 | 91 ± 8 (NS) | 1467 ± 398 | 101 ± 12 (NS) |
| 20 min | 7887 ± 283 | 68 ± 2 (**) | 3828 ± 347 | 77 ± 5 (*) | 1353 ± 416 | 96 ± 9 (NS) |

FIG. 6. Effect of AACOCF3 and BEL on the release of [3H]arachidonic acid in rat submandibular ductal cells stimulated with Bz-ATP. [3H]AA-labeled ductal cells were washed and resuspended in a magnesium-free HBSS medium containing 0.125% BSA and 0.5 mM CaCl2 (●) or in the presence of 0.5 mM EGTA (○). Cells were preincubated for 2 min with various concentrations of AACOCF3 (left panel) or for 5 min with various concentrations of BEL (right panel). The reaction was carried out at 37 °C and stopped by centrifugation 20 min after stimulation. The radioactivity of the supernatants was measured, and the results are expressed as percentage of radioactivity with respect to unstimulated controls. Data represent mean ± S.E. of three to six independent experiments performed in triplicate.

extracellular calcium, 30 μM BEL fully inhibited the residual response to Bz-ATP (Fig. 6, right panel).

BEL and AACOCF3 were also used to test the role of the iPLA2 and the cPLA2 in the remodeling of cellular phospholipids. RSMG ductal cells were preincubated for 5 min either in basal conditions or in the presence of 100 μM BEL or AACOCF3 and then incubated in the same conditions but in the presence of [3H]AA. After 60 min, the radioactivity present in the phospholipid fraction was quantified. The presence of BEL and AACOCF3 decreased, respectively, 90 ± 1% and 71 ± 3% the incorporation of [3H]AA in the phospholipids (data not shown). These data suggest that iPLA2 (which is not specific for arachidonyl-containing phospholipids) has a constitutive activity which participates in the selective incorporation of arachidonic acid in phospholipids.

Lack of Correlation between the Activation of a Phospholipase C and the Release of [3H]AA—As reported previously, ATP- and Bz-ATP-stimulated [3H]AA release could reflect phospholipase C activation by the purinergic receptors and subsequent stimulation of a calcium- or protein kinase C-dependent phospholipase A2 (26, 30). In order to determine the relationship between the [3H]AA release and the phospholipase C activation, the cells were labeled with myo-[2-3H]inositol. The stimulatory effect of Bz-ATP on [3H]AA release from ducts was not secondary to the activation of phospholipase C, since 100 μM Bz-ATP, which was the best agonist on [3H]AA release, did not significantly increase the liberation of inositol phosphates; the radioactivity present in the fraction containing IP1+IP2+IP3 increased from 200 ± 47 dpm in the absence of Bz-ATP to 263 ± 73 dpm in its presence (p < 0.01). At the opposite, 100 μM carbachol, which did not significantly increase the release of [3H]AA (Table I), increased the radioactivity present in the inositol phosphate fraction to 1,366 ± 152 dpm (p < 0.001 when compared with control). These results confirmed that the increase in the [Ca2+]i through the phospholipase C activation was not responsible for the Bz-ATP-stimulated [3H]AA release.

Role of the Two Phospholipases A2 in the Physiology of the Ductal Cells—It has been reported previously that the activation of ionotropic receptors increases the release of kallikrein by RSMG ductal cells (18). The role of the cPLA2 and iPLA2 in this response was tested next (Fig. 7). The ducts were preincubated with 100 μM AACOCF3 or BEL in a medium containing 0.5 mM CaCl2, and the release of kallikrein in the medium was measured after incubation either in basal conditions or in the presence of 100 μM Bz-ATP. The two inhibitors had divergent effects on the secretion of kallikrein both in basal conditions and in the presence of the purinergic agonist. AACOCF3 increased the basal release of kallikrein from 18 ± 2% to 50 ± 7% (n = 3). It also enhanced the secretory response to Bz-ATP. Bz-ATP increased the secretion of kallikrein to 42 ± 1% in the absence of AACOCF3 and to 75 ± 4% in its presence. Taking into account that AACOCF3 is a structural analogue of AA, we tested whether exogenous AA could also increase kallikrein secretion. At 1, 10, and 100 μM, AA had no effect on the secretion of kallikrein. BEL was a strong inhibitor of kallikrein secretion, which decreased to 3 ± 1% in basal conditions and to 6 ± 2% in the presence of Bz-ATP (Fig. 7). The inhibitory effect of BEL was dose-dependent (Fig. 8). Half-maximal inhibitory concentrations were 10 μM both on basal and on Bz-ATP-stimulated kallikrein release. Considering that BEL was originally designed to inhibit the serine protease α-chymotrypsin (44), BEL could inhibit the activity of kallikrein rather than inhibit its secretion. To test this hypothesis, RSMG ductal cells were homogenized and the esterase activity of the homogenate was measured in the absence or in the presence of increasing concentrations of BEL. At a 100 μM concentration, the inhibition exerted by BEL on the activity of kallikrein never exceeded 15% (data not shown). These results confirmed that BEL inhibited the secretion of kallikrein rather than the activity of the enzyme. The involvement of an iPLA2 in the secretory response to Bz-ATP was further confirmed by measuring the release of kallikrein in the absence of extracellular calcium. In this condition, the basal release of kallikrein was not significantly affected and the secretory response to Bz-ATP was decreased from 46 ± 2% in the presence of 0.5 mM calcium to 36 ± 4% in the presence of 0.5 mM EGTA. The response was still highly significant when compared with basal release (p < 0.01, n = 4).

DISCUSSION

The present results constitute the first demonstration by RT-PCR of the expression of P2X7 in ductal cells of RSMG. This receptor is coupled with the activation of two distinct phospho-
lipases A2. Indeed, ATP and Bz-ATP increased the release of [3H]AA from prelabeled RSMG ductal cells. Based on the response to various agonists, on the sensitivity to antagonists like Coomassie Blue, or on the effect of divalent cations on the response to ATP, two types of purinergic receptors on RSMG ductal cells have been described (18). At low concentrations, ATP stimulated a G-protein-coupled P2Y1 receptor that was very sensitive to 2-MeSATP. The occupancy of this receptor increased the intracellular concentration of IP3, and an intracellular pool of calcium was mobilized. The removal of calcium from the extracellular medium or the addition of magnesium did not suppress this response to ATP. This receptor is present in freshly isolated RSMG ductal cells, but its coupling with an effector disappears during cell cultures (22). At high concentrations, ATP and its analog Bz-ATP both induced a massive increase of the [Ca^{2+}]), which was fully dependent on the presence of calcium in the extracellular medium (18). This response was blocked by Coomassie Blue or by the decrease of the concentration of ATP4 secondary to the addition of magnesium to the medium. The secretion of kallikrein by ductal cells was increased in response to P2Z agonists (18). As shown in this work, ATP and Bz-ATP, two agonists of the P2Z receptors, increased the release of [3H]AA from RSMG ductal cells. This response was not reproduced by agonists of the P2Y receptors. The release of [3H]AA was not secondary to the activation of a polyPL-specific phospholipase C since an agonist like carbachol, which activated this enzyme, had no significant effect on the release of [3H]AA. It was inhibited by oATP, suramin, or Coomassie Blue, antagonists of the P2Z Receptors. The presence of magnesium in the medium also suppressed the release of [3H]AA in response to ATP and Bz-ATP, further confirming that P2Z receptors were involved in this response. Nickel also inhibited the response to the purines. We could calculate that at concentrations of nickel as high as 1 mM, the concentration of ATP4 in the medium should be sufficient to fully stimulate the P2Z receptors. RSMG ductal cells were impermeant to nickel since the ion did not quench the fluorescence of intracellular Fura-2 and since the inhibition by nickel was instantaneously reversed by the addition of DTPA, a non-permeant chelator of the ion. The inhibition by nickel could thus only be explained by a direct blockade by this cation of the nonspecific cation channel coupled to P2Z receptors. From these results, it could be concluded that the activation of the nonspecific cation channels coupled to P2Z receptors increased the release of [3H]AA from RSMG ductal cells.

The [3H]AA released in the medium in response to purinergic agonists originated from the cellular phospholipids, mainly PC. Indeed, the decrement in the content of [3H]AA of this class of phospholipids was in agreement with the increment of the [3H]AA released into the extracellular medium. Several lipases could be involved in the release of [3H]AA from prelabeled phospholipids. It has been reported previously that P2Z agonists can activate a phospholipase D in BAC1.2F5 macrophages (27) or in lymphocytes (29). The phosphatidic acid generated by this enzyme could be hydrolyzed by a phospholipase A2 or could generate diglycerides after hydrolysis by a phosphatidate-phosphohydrolase. A diglyceride lipase would then liberate [3H]AA from these diglycerides. Such a pathway has been described in peritoneal mast cells (45) and accounts for the release of [3H]AA in response to muscarinic agonists in RSMG acinar cells (46). Preincubation of [3H]AA-labeled RSMG ductal cells with propranolol (an inhibitor of the phosphatidate-phosphohydrolase, 47) did not affect the subsequent ATP- or Bz-ATP-release of [3H]AA (data not shown). The release of [3H]AA was inhibited by inhibitors of PLA2. These results suggest that a PLA2 and not a diglyceride lipase is responsible for the release of [3H]AA. At the present time, we are unable to exclude the activation of a phospholipase D previous to the activation of PLA2. It should, however, be mentioned that: 1) the purines did not increase the release of free choline from phospholipids labeled with [3H]choline; and 2) the activation of PLD by P2Z agonists is dependent on bivalent cation influx (29), but the release of [3H]AA in response to ATP could be observed in the absence of divalent cation in the medium and in the presence of EGTA, a chelator of these cations.

Two major groups of intracellular PLA2 have been described. The calcium-dependent cytosolic PLA2 (cPLA2) has a molecular mass of 85 kDa. This enzyme is rather specific for 2-arachidonoyl phospholipids. The NH2 terminus of this enzyme is a calcium-binding domain analogous to the C2 domain of protein kinase C. This enzyme cannot be activated in the absence of an increase of [Ca^{2+}]. After interacting with the C2 domain of the...
enzyme, calcium promotes its translocation from the cytosol to intracellular membranes, bringing the calcium-independent catalytic site to the membrane substrate (48). Various calcium-independent phospholipases A2 have been described (49). Ankyrin domains have been described in these iPLA2, which could explain their self-association to form catalytically competent species and their interaction with proteins that may regulate their catalytic properties (50). Considering that no inhibitor is fully specific of the iPLA2, the release of [3H]AA in the absence of extracellular calcium is the best evidence for the involvement of this enzyme in the hydrolysis of cellular phospholipids (49). In our hands, the removal of extracellular calcium inhibited but did not suppress the release of [3H]AA in response to Bz-ATP. This result suggested that P2X7 receptors could activate both calcium-dependent and calcium-independent phospholipases A2. This was further confirmed by the use of AACOCF3 (an inhibitor of cPLA2) and BEL (an inhibitor of iPLA2). Neither of these inhibitors is fully specific; it has been claimed that AACOCF3 could inhibit iPLA2 (49, 51) and that BEL could inhibit the phosphatidate phosphohydrolase (52). In the presence of extracellular calcium, none of these two inhibitors could completely block the release of [3H]AA. The inhibition exerted by AACOCF3 was not observed in the absence of extracellular calcium, confirming that this inhibitor blocked a calcium-sensitive PLA2. The presence of a cPLA2 in RSMG ductal cells was confirmed by Western blots using commercially available rabbit polyclonal antibodies (data not shown). The poor quality of the gels (due to the high background) precluded any study of the phosphorylated state of the enzyme. We were also unable to demonstrate the translocation of the enzyme from the cytosol to a membrane compartment in the presence of Bz-ATP (data not shown). The inhibition exerted by BEL on the release of [3H]AA was still observed in the absence of extracellular calcium; the stimulation of [3H]AA release by Bz-ATP was completely blocked by a combination of calcium removal and BEL. This confirmed that P2X7 agonists activated an iPLA2. The mechanisms involved in the activation of iPLA2 remain poorly understood. The enzyme has several ankyrin domains, which are involved in the interaction with other proteins like phosphofructokinase or in the interaction between several monomers of iPLA2 (50). It has indeed been reported that the active form of the enzyme has an oligomeric structure. More recently, it was shown that the splicing of the introns can activate the enzyme (51). It has been recently reported that in macrophages, P2X7 agonists activate a transcription factor, NF-κB (54). Like iPLA2, this protein has several ankyrin domains (55), and splice variants of a precursor behave as antagonists (54). The activation of iPLA2 and NF-κB by P2X7 agonists might involve similar mechanisms like activation of the ubiquitin-proteosome pathway (56).

Considering that P2X7 receptors activate both a cPLA2 and an iPLA2 and promote the secretion of kallikrein, the correlation between these responses remained to be established. BEL was a strong inhibitor of both basal and Bz-ATP-stimulated release of kallikrein. Half-maximal concentrations of BEL on [3H]AA release and on kallikrein secretion were similar, suggesting that the two responses were coupled. Furthermore, N122 was able to block in a dose-dependent manner the Bz-ATP-stimulated kallikrein secretion (data not shown). These results suggest that iPLA2 was responsible for the release of kallikrein in response to purinergic agonists. The contribution of this phospholipase A2 to amylase secretion from rat parotid acini (57) and to insulin secretion by pancreatic β cells (58) has also been reported. This is consistent with the fact that calcium is not required for the fusion of secretory granules with the plasma membrane (59). Additionally, the presence of an iPLA2 on the membrane of rat parotid secretory granules has been reported (60). The stimulation by AACOCF3 of kallikrein secretion by RSMG ductal cells (this report) or of amylase by rat parotid acini (57) is more difficult to interpret. This response was observed at concentrations higher than 10 μM. These concentrations have no lytic effect (measured by the release of lactate dehydrogenase or the release of Fura-2, data not shown). Considering that 1) the activation of the two phospholipases A2 and the hydrolysis of PC did not lead to an accumulation of intracellular lysophospholipids, 2) lysophospholipids are known fusogens, 3) cPLA2 is a strong lysophospholipase (it was recently shown that cPLA2 could hydrolyze both sn1 and sn2 isomers of palmitoylglycerol-3-phosphocholine; Ref. 61), 4) AACOCF3 is an inhibitor of the lysophospholipase activity of cPLA2 (61), 5) the major role of iPLA2 would be the remodeling of phospholipids in membranes (49), and 6) macrophages expressing P2X7 receptors spontaneously fuse (62), we would like to propose the following model. The iPLA2 would be activated by P2X7 agonists. This enzyme, which has a weak specificity for arachidonyl-containing phospholipids, would release free fatty acids and sn1-acyl lysophospholipids (probably sn1-acyl lysophosphorylcholine). The lysophospholipids would favor the fusion between the membrane of the secretory granule and the apical plasma membrane. These lysophospholipids could be metabolized in two ways. 1) They might be reacylated to phospholipids (the Landa cycle) by an acylase specific for arachidonic acid. This would explain why a preincubation of the RSMG ductal cells with BEL abolished the incorporation of [3H]AA in the cellular phospholipids. 2) The lysophospholipids might be hydrolyzed by the lysophospholipase activity of cPLA2. According to this second hypothesis, the secretory response to AACOCF3 could be secondary to the accumulation of lysophospholipids generated by the constitutive activity of iPLA2 and the inhibition of cPLA2 by AACOCF3. However, other hypotheses cannot be excluded. It has been shown that AACOCF3 can be reduced to AACOCH3, which, by itself, increased the stimulated level of AA in cell-based assay and the production of 12-hydroxyeicosatetraenoic acid by ionophore-stimulated platelets (63). The increased level of AA in response to these metabolites could trigger exocytosis. Another hypothesis would be that AACOCF3 behaves as an analog of AA and stimulates the secretory process, although in our hands the presence of AA did not affect the basal secretion of kallikrein.

In conclusion, P2X7 agonists increase the release of [3H]AA from RSMG ductal cells by activating two phospholipases A2, a cPLA2 and an iPLA2. The activation of the iPLA2 is responsible for the secretion of kallikrein by ductal cells in response to P2X7 activation.

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REFERENCES

1. Harden, T. K., Boyer, J. L., and Nicholas, R. A. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 541–579
2. Weisman, G. A., Turner, J. T., and Fedan, J. S. (1996) J. Pharmocol. Exp. Ther. 277, 1–9
3. Abracchio, M. P., and Burnstock, G. (1994) Pharmacol. Ther. 64, 445–475
4. Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., Surprenant, A., and Buell, G. (1994) Nature 371, 516–519
5. Lewis, C., Neidhart, S., Holy, C., North, R. A., Buell, G., and Surprenant, A. (1995) Nature 377, 432–435
6. Buell, G., Lewis, C., Collo, G., North, R. A., and Surprenant, A. (1996) J. Neurosci. 16, 55–62
7. Collo, G., North, R. A., Kawashima, E., Merlo-Pich, E., Neidhart, S., Surprenant, A., and Buell, G. (1996) J. Neurosci. 16, 2495–2507
8. Surprenant, A., Rassendren, F., Kawashima, E., North, R. A., and Buell, G. (1996) Science 272, 735–738
