Coupling of two pools of P2X$_7$ receptors to distinct intracellular signaling pathways in rat submandibular gland[s]

Mikel Garcia-Marcos,* Encarnación Pérez-Andrés,* Séverine Tandel,[†] Unai Fontanils,*
Alain Kumps,[§] Elie Kabré,** Antonio Gómez-Muñoz,* Aida Marino,*
Jean-Paul Dehaye,[1,†] and Stéphanie Pochet[†]

Departamento de Bioquimica y Biologia Molecular,* Facultad de Ciencias, Universidad del Pais Vasco,
48080 Bilbao, Spain; Laboratoire de Biochimie et de Biologie Cellulaire,[†] and Laboratoire de Chimie
Médicale,[§] Institut de Pharmacie C.P. 205/3, Université libre de Bruxelles, B-1050 Brussels, Belgium; and
Laboratoire de Biochimie et d'Immunologie,** Unité de Formation et de Recherche des Sciences de la
Santé, Université de Ouagadougou, Burkina Faso

Abstract The plasma membrane of cells from rat submandibular glands was isolated and extensively sonicated. The homogenate was centrifuged at high speed in a discontinuous sucrose gradient. Light fractions contained vesicles analogous to rafts: they were rich in cholesterol, they contained GM1 and caveolin-1, and P2X$_7$ receptors were detected in these fractions. The location of the P2X$_7$ receptors in rafts was abolished when cellular cholesterol was removed by methyl-β-cyclodextrin (MCD). ATP activated neutral sphingomyelinase (N-SMase), which provoked a decrease of the cellular content of sphingomyelin and an increase of ceramide levels in these cells and in the rafts. Treatment with MCD and filipin (but not with α-cyclodextrin) abolished the increase of the intracellular concentration of calcium ($[Ca^{2+}]_i$) in response to epinephrine but not to ATP. MCD and filipin also inhibited the activation by ATP of phospholipase A$_2$ (PLA$_2$). Inhibition of N-SMase with glutathione or GW4869 prevented the activation of PLA$_2$ by P2X$_7$ agonists without affecting $[Ca^{2+}]_i$ levels. We conclude that P2X$_7$ receptors are present in both raft and nonraft compartments of plasma membranes; the receptors forming a nonselective cation channel are located in the nonraft fraction. P2X$_7$ receptors in the rafts are coupled to the activation of N-SMase, which increases the content of ceramides in rafts. This may contribute to the activation of PLA$_2$ in response to P2X$_7$ receptor occupancy.— Garcia-Marcos, M., E. Pérez-Andrés, S. Tandel, U. Fontanils, A. Kumps, E. Kabré, A. Gómez-Muñoz, A. Marino, J-P. Dehaye, and S. Pochet. **Coupling of two pools of P2X$_7$ receptors to distinct intracellular signaling pathways in rat submandibular gland.** *J. Lipid Res.* 2006. **47:** 705–714.

Supplementary key words calcium • methylcyclodextrin • rafts • ceramides • purinergic • salivary glands • sphingomyelinase

Purinergic receptors are expressed by acini and ducts, both in parotid and submandibular glands (1). These receptors, which belong to the P2 family of purinergic receptors, have been divided into two major classes (2). The P2Y receptors have seven transmembrane domains and are coupled to a regulatory GTP binding protein. The P2X receptors have two transmembrane domains separated by ∼300 amino acids, which make a large extracellular loop (3). Both the N and C termini are probably cytoplasmic. The extracellular loop contains several conserved *N*-glycosylation sites and 10 cysteine residues forming five disulfide bridges (4). These receptors are not coupled to a regulatory protein, but when activated by an agonist they form a cationic channel. It has been suggested that at least three subunits, identical or different, must assemble in the membrane to form the channel (3).

Salivary glands express mostly P2X$_7$ receptors (5), which are rather unique among the P2X receptors. Although the six other P2X receptors have ∼450 amino acids, the P2X$_7$ receptor is 595 amino acids long (6). A C terminus that is 120 amino acids longer than that of the other P2X receptors accounts for this difference in size (3). The very long (240 amino acids) intracellular C terminus of the P2X$_7$ receptor confers to this protein unexpected properties, such as the capacity to form a pore (7). After pro-

longed exposure to high concentrations of ATP, cells become permeant to charged molecules, such as ethidium[+] or propidium[+].

The formation of this pore can even lead to cell death (8). In fact, this receptor can trigger apoptosis (9) as a consequence of either massive uptake of chloride (10) or leakage of intracellular potassium (11). Prolonged activation of the P2X[7] receptor provokes major cellular changes, such as fragmentation of DNA (12) or vesiculation and blebbing of the plasma membrane (13). The role of the intracellular C terminus in the pore-forming activity of the P2X[7] receptor has been confirmed by the expression of truncated receptors in HEK293 cells. Removal of the last 177 amino acids abolishes the capacity of the protein to form a pore without affecting its ability to form a channel (7).

This observation has stimulated the search for specific domains in this region of the receptor. Denlinger et al. (14) reported that residues 436–531 of the P2X[7] receptor are similar to a region that overlaps with the death domain of tumor necrosis factor receptor 1 (TNFR1). This similarity of structure might explain why tumor necrosis factor (TNF) and ATP share the ability to activate caspases and induce apoptosis. Proteins expressing the death domain can generate homotropic interactions (15), and it has been shown that such interactions target the TNFR1 to specific microdomains of the plasma membrane, the so-called lipid rafts (16). These domains are favored by their high content of saturated phospholipids, cholesterol, and sphingolipids (17). This lipid enrichment can explain their rigidity in a membrane that, according to the model of Singer and Nicolson (18), is a fluid mosaic.

The purpose of our work was thus to examine the presence of the P2X[7] receptors in these microdomains. Our results show that P2X[7] receptors are concentrated in raft microdomains. We also report that the stimulation of the cells with ATP increases their ceramide content by activating neutral sphingomyelinase (N-SMase). We present evidence that the formation of the nonselective cation channel by P2X[7] receptors [unlike their activation of phospholipase A[2] (PLA[2])] does not require the integrity of the raft domains. Finally, we show that the activation of N-SMase is involved in the activation of PLA[2] in response to ATP. Some of these results have been published in abstract form (19).

EXPERIMENTAL PROCEDURES

Preparation of cellular fractions

Cells were isolated from rat submandibular glands as described previously (20). Isolation of lipid rafts was performed according to the method described by Liu, Casey, and Pike (21). The cellular pellet was resuspended in 1 ml of TEEA hypotonic buffer (20 mM Tris, 1 mM EGTA, 1 mM EDTA, and a cocktail of inhibitors of proteases containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM PMSF, pH 8.0) and passed 10 times through a 29G needle. After a 10 min centrifugation at 1,000 g at 4°C, the supernatant was saved and the pellet was extracted by the same procedure. This process was repeated four times, and the pooled supernatants were centrifuged at 100,000 g for 30 min at 4°C in a SW60Ti rotor. The crude membranes obtained in the pellet were resuspended in 800 μl of HEPES-saline buffer (25 mM HEPES and 150 mM NaCl, pH 7.4) containing the cocktail of protease inhibitors and then sonicated on ice four times for 20 s at 10–12 μm amplitude. This membrane homogenate was mixed with an equal volume of 90% sucrose. Gradients were prepared in centrifuge tubes by the successive addition of 1.4 ml of the membrane homogenate/sucrose mixture, 1.8 ml of 35% sucrose, and finally 1 ml of 5% sucrose. The sucrose solutions were prepared in HEPES-saline medium. The tubes were centrifuged for 16 h at 200,000 g in a SW60Ti rotor. Fractions of 400 μl were removed from the top to the bottom of the gradient (fractions 1–10) and kept on ice.

Characterization of the cellular fractions

The light-scattering assay, the measurement of cholesterol, proteins, or GM1 gangliosides, the fluidity of the membrane, the detection of proteins by immunoblotting, and the measurement of the intracellular concentration of calcium ($[Ca^{2+}]_i$) have been described previously (22). For immunoblotting, the antibody dilutions from manufacturer stock and final concentrations were as follows: P2X[7], 1:666 (0.45 μg/ml); caveolin-1, 1:1,250; transferrin receptor (TfR), 1:250 (4 μg/ml); β-COP (β-coatomer protein), 1:750 (4 μg/ml); calnexin, 1:1,000; horseradish peroxidase-conjugated anti-mouse IgG, 1:2,000 (0.5 μg/ml); horseradish peroxidase-conjugated anti-rabbit IgG, 1:10,000. To avoid the fluorescent interference of GW4869 and filipin, some calcium assays were performed by loading the cells with 4 μM fluo-4/AM instead of fura-2/AM. In this case, the excitation and emission wavelengths were 495 ± 4 and 515 ± 4 nm, respectively, and the results were normalized taking maximal and minimal fluorescent signals as references (normalized arbitrary units). The activity of PLA[2] was measured as described previously (23). Briefly, the activity was estimated as the relative release of [³H]oleic acid after 10 min of stimulation with 1 mM ATP compared with non stimulated samples.

Drug treatment conditions

Myriocin and GSH were used at 25 μM and 5 mM, respectively, for 60 min at 37°C, and GW4869 was used at 20 μM for 30 min at 37°C before stimulation with 1 mM ATP. Cells were preincubated with 25 mM methyl-β-cyclodextrin (MCD) or 25 mM α-cyclodextrin (ACD) for 10 min or with 15 μM filipin for 30 min at 37°C. In the latter conditions, the cells were washed before ATP stimulation.

Measurement of sphingomyelinase activity

Sphingomyelinase activity was assayed as described by Liu and Hannun (24). Cells from rat submandibular glands were prepared as described previously and resuspended in HEPES-buffered saline (HBS) medium (24.5 mM HEPES, pH 7.4, 96 mM NaCl, 6 mM KCl, 1 mM MgCl[2], 2.5 mM NaH[2]PO[4], 11.5 mM glucose, 5 mM sodium pyruvate, 5 mM sodium glutamate, and 5 mM sodium fumarate) supplemented with 0.5 mM CaCl[2]. Aliquots of 500 μl were incubated at 37°C in the absence or presence of 1 mM ATP for the indicated times. Cells were centrifuged at 500 g, the supernatant was discarded, and the pellets were washed once with ice-cold isotonic NaCl. The pellets were resuspended in 200 μl of lysis buffer, and cells were disrupted by three cycles of freezing and thawing in a methanol-dry ice bath. For N-SMase, the lysis buffer consisted of 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 5 mM 3-glycerolphosphate,

1 mM sodium fluoride, 1 mM sodium molybdate, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. For acidic sphingomyelinase (A-SMase), the lysis buffer Tris-HCl was substituted by 50 mM sodium acetate, pH 5.0, and DTT was removed. The lysate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was saved for the determination of sphingomyelinase activity. Fifty microliters of this supernatant containing 75–100 μg of protein for N-SMase or 30–40 μg of protein for A-SMase was mixed with 50 μl of the substrate micelles. The reaction mixture consisted of 100 mM Tris-HC1, pH 7.4, 5 nmol of [^{14}C]sphingomyelin (100,000 dpm per reaction), 5 nmol of phosphatidylserine, 5 mM DTT, 0.1% Triton X-100 (1.54 mM), 5 mM magnesium chloride, and enzyme sample in a final volume of 100 μl. For A-SMase, Tris-HCl was substituted by sodium acetate, pH 5.0, and no phosphatidylserine, DTT, or magnesium chloride was added. The enzymatic reaction was carried out at 37°C for 90 min for N-SMase and for 10 min for A-SMase. It was stopped by the addition of 1.5 ml of chloroform-methanol (2:1) and 0.2 ml of water. After vortexing and phase separation by centrifugation at 1,500 g for 5 min, a portion (0.2 ml) of the upper aqueous phase, containing released phosphorylcholine, was removed and mixed with 2 ml of scintillation cocktail and counted in a LKB Wallac MiniBeta scintillation counter (LKB, Ghent, Belgium). Enzyme activities were expressed as nanomoles of substrate hydrolyzed per milligram of protein per hour and were normalized to control values.

Ceramide determination

Cells were incubated for 30 min at 37°C in HBS medium supplemented with 0.5 mM CaCl$_2$ in the absence or presence of 1 mM ATP. At the end of the incubation, lipids were extracted (25) or the raft and nonraft fractions were prepared before lipid extraction. One milliliter of methanol and 1 ml of chloroform were added to 0.9 ml of the lipid aqueous suspension. The mixture was vortexed and centrifuged at 1,000 g for 5 min to allow separation of the two phases. The organic bottom phase was washed twice with an aqueous solution (2 M KCl/0.2 M HCl) saturated with methanol and chloroform. The upper phase was discarded, and the lower phase was kept for the assays. Eight hundred microliters was vacuum-dried in a Savant SpeedVac AS290 concentrator for ceramide determination, and 80 μl was kept for the determination of phosphates (26). Ceramide was quantified by the diacylglycerol kinase assay (27). Each dried sample or ceramide standard was incubated in 0.1 ml of the reaction mixture [1.2 mM diethylenediamine pentaacetic acid, 12.5 mM MgCl$_2$, 50 mM NaCl, 1 mM EGTA, 50 mM imidazole, 10 mM dithiothreitol, 1 mM ATP, 1 mM cardiolipin, 1.5% (w/v) N-octyl-β-D-glucopyranoside, and ≥10 mU of diacylglycerol kinase from *Escherichia coli*]. This reaction mixture was preincubated for at least 20 min at 37°C to phosphorylate any trace of ceramide in the solution of diacylglycerol kinase of bacterial origin. Then, 1 μCi of [γ-^{32}P]ATP was added to each tube, and the reaction was carried out for 90 min at 37°C. The reaction was stopped by addition of the solvents of the lipid extraction. Lipids were extracted and dried as described above. They were resuspended in 50 μl of chloroform and spotted onto a TLC plate. The plate was developed consecutively with two mixtures of solvents. The first eluent was a mixture of chloroform-methanol-ammonium hydroxide (65:35:7.5, v/v), and the second eluent was a mixture of chloroform-methanol-acetic acid-acetone-water (50:10:10:20:5, v/v). The radioactive spots were visualized by exposure to radiographic film and scraped into scintillation vials. Ceramides in the samples were determined by comparison with a standard curve and normalized to the quantity of phosphorus.

It should be noted that the various treatments did not affect the cellular phosphorus content.

Sphingomyelin determination

Cells from one rat submandibular gland were resuspended in 3 ml of HBS medium in the presence of 0.5 mM CaCl$_2$. They were incubated for 90 min at room temperature in the presence of 6 μCi/ml [^{3}H]palmitic acid and 3 μCi/ml [^{3}H]choline. At the end of the labeling period, the cells were washed three times with isotonic NaCl. The final pellet was resuspended in 3 ml of fresh HBS medium without the tracer and incubated for 30 min at room temperature. After this wash-out incubation, the cells were washed twice with isotonic NaCl and resuspended in fresh HBS medium with 0.5 mM CaCl$_2$ and 0.5% BSA. Aliquots of 200 μl were incubated in a final 500 μl of HBS medium with 0.5 mM calcium and 0.5% albumin in the absence or presence of an agonist for the various times at 37°C. The lipids were extracted as described above and resuspended in 50 μl of chloroformic solution containing cold sphingomyelin (15 μg/tube). Lipids were spotted onto a TLC plate and developed in the upper phase of a mixture of chloroform-methanol-acetic acid-water (75:45:12:3, v/v). The lipids were visualized by exposure to iodine vapor, and sphingomyelin was identified by comparison with an authentic standard. The spots were scraped into scintillation vials containing 0.2 ml of methanol. Two milliliters of scintillation liquid was added to each vial, and their radioactivity was determined by β liquid scintillation. Sphingomyelin was expressed as a percentage of the radioactivity in controls.

Statistical analysis

In experiments that were repeated at least three times, the results are expressed as means ± SEM of the number of experiments indicated. When experiments were repeated only twice, the results of the each experiment are presented or the results are presented as means with range. Statistical significance between various conditions was assessed with Student's t-test.

Materials

Male Wistar rats (150–200 g) were purchased from Charles River Laboratories (Brussels, Belgium). The housing and care of the animals were in agreement with the regulations of the European Union. The animals were fed ad libitum with free access to water. Fura-2/AM and fluo-4/AM were from Molecular Probes (Eugene, OR). BSA (fraction V) was from Roche (Mannheim, Germany), and the glutamine-free amino acids mixture was from Gibco BRL (Paisley, Scotland). Epinephrine, MCD, ACD, digitonin, filipin, HEPES, diacylglycerol kinase, N-octyl-β-D-glucopyranoside, 2′-3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (Bz-ATP), GSH, DTT, myriocin, diethylenediamine pentaacetic acid, cardiolipin, sphingomyelin, phosphatidylserine, and the anti-β-COP mouse monoclonal antibody (clone maD) were obtained from Sigma (St. Louis, MO). 9,10-[^{3}H]oleic acid was from American Radiochemical Co. (St. Louis, MO), [^{3}H]palmitic acid and [^{3}H]choline, [γ-^{32}P]ATP, horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG, and chemiluminescence reagents (ECL+) were from Amersham Biosciences (Piscataway, NJ). The anti-P2X$_7$ rabbit polyclonal antibody was from Alomone (Jerusalem, Israel). The anti-TfR mouse monoclonal antibody (clone OX-26) was from Biogenesis (Poole, England), and the anti-calnexin rabbit polyclonal antibody was from Stressgen (Victoria, Canada). The scintillation solution Ecoscint A was from National Diagnostics (Atlanta, GA). GW4869 was obtained from Calbiochem (Palo Alto, CA) and was stored and used exactly as described (28).

RESULTS

Presence of P2X$_7$ receptors in two fractions of the plasma membrane

Rafts were isolated after extensive sonication of the plasma membrane followed by centrifugation on a discontinuous sucrose gradient. As shown in **Fig. 1A**, light scattering was observed not only in heavy fractions (7–10) but also in fractions corresponding to the 5–35% sucrose interface (2–4), suggesting that they contained vesicles. These low-density fractions of the gradient contained cholesterol (Fig. 1B) and a small amount of protein (Fig. 1C), including caveolin-1 (Fig. 1E). These fractions bound the B-subunit of cholera toxin (Fig. 1D), indicating that they contained ganglioside GM1. Calnexin (a marker for the endoplasmic reticulum) and β-COP (a marker for the Golgi apparatus) were not detected in low-density fractions (Fig. 1H, 1G). Only a small fraction (~10%) of the TfR (a protein located in the nonraft fraction of the plasma membrane) was detected in the light fractions (Fig. 1F) (29). Fractions 2–4 and 6–10 were pooled, and their fluidity was estimated by measuring anisotropy. At each temperature (in the 15–40°C range), the anisotropy of lighter fractions was higher than the anisotropy of heavier fractions, suggesting that the lighter fractions were more rigid (data not shown). All these results con-
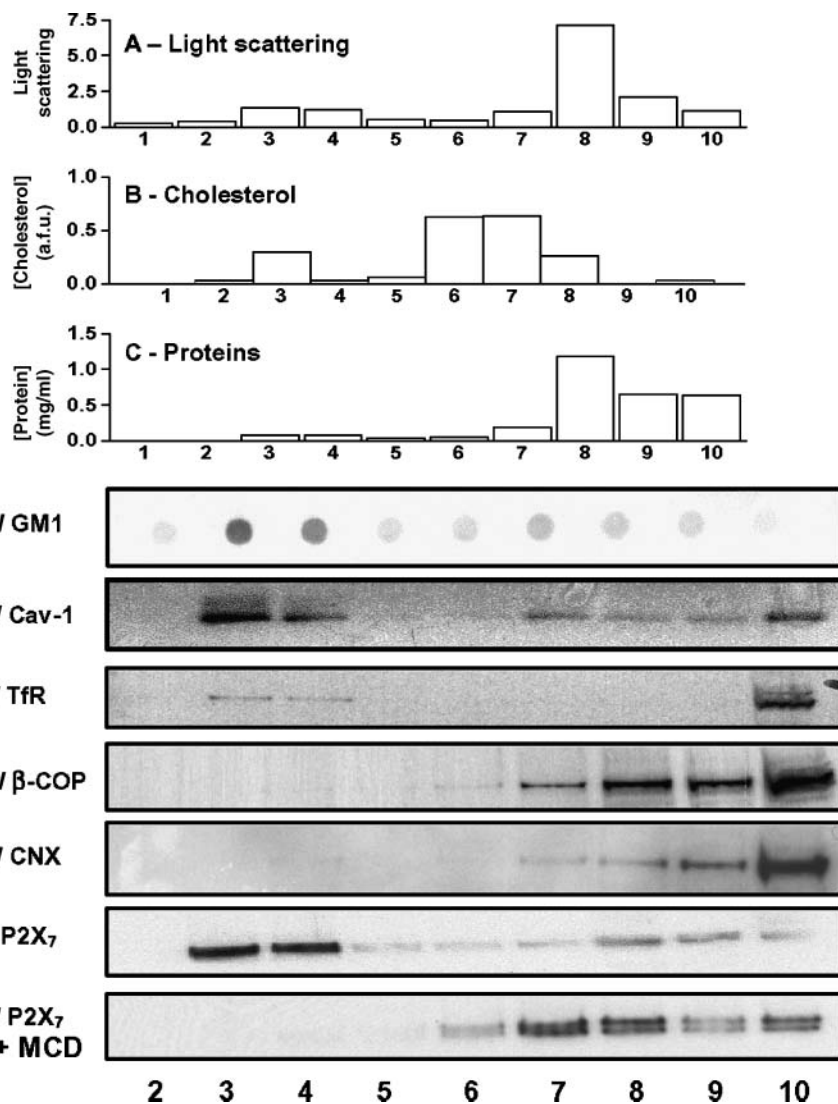

Fig. 1. Characterization of the membrane fractions prepared by sonication from rat submandibular glands. Raft and nonraft membranes were prepared by sonication followed by centrifugation in a discontinuous sucrose gradient. Ten fractions were collected (fraction 1, top of the gradient; fraction 10, bottom of the gradient). Each fraction was tested for light scattering (A), cholesterol content (B), or protein content (C). They were also tested by dot blots for ganglioside GM1 (D) and by Western blots for caveolin-1, transferrin receptor (TfR), β-COP (β-coatomer protein), calnexin (CNX) and P2X$_7$ receptors (panels E, F, G, H, and I respectively). Panel J represents the distribution of P2X$_7$ receptors in fractions from cells incubated in the presence of 25 mM methyl-β-cyclodextrin (MCD) for 10 min at 37°C. The results are representative of three to six experiments. Afu, arbitrary fluorescence units.

firmed that fragments of the plasma membrane with properties comparable to rafts could be isolated by sonication. The fractions obtained by sonication were tested for P2X$_7$ receptors. As shown in Fig. 1I, two different populations of receptors were observed: one population migrated with rafts, and the other population migrated with the heavier fractions of the sucrose gradient.

Effect of raft disruption on the responses to extracellular ATP

Preincubation of submandibular gland cells with MCD removes plasma membrane cholesterol and disrupts the rafts (22). Activation of P2X$_7$ receptors increases the $[Ca^{2+}]_i$ and enhances PLA$_2$ activity. Considering that a fraction of

P2X$_7$ receptors are located in rafts, we examined whether raft disruption by MCD would interfere with these responses to P2X$_7$ agonists. In a first experiment, the effect of MCD on the distribution of the P2X$_7$ receptors in the plasma membrane was considered. No P2X$_7$ receptor immunoreactivity was detected in rafts isolated from MCD-treated cells (Fig. 1J). As shown in the upper panel of **Fig. 2**, treatment with MCD had no effect on the variation of $[Ca^{2+}]_i$ in response to 1 mM ATP [from 94 ± 16 nM in control cells to
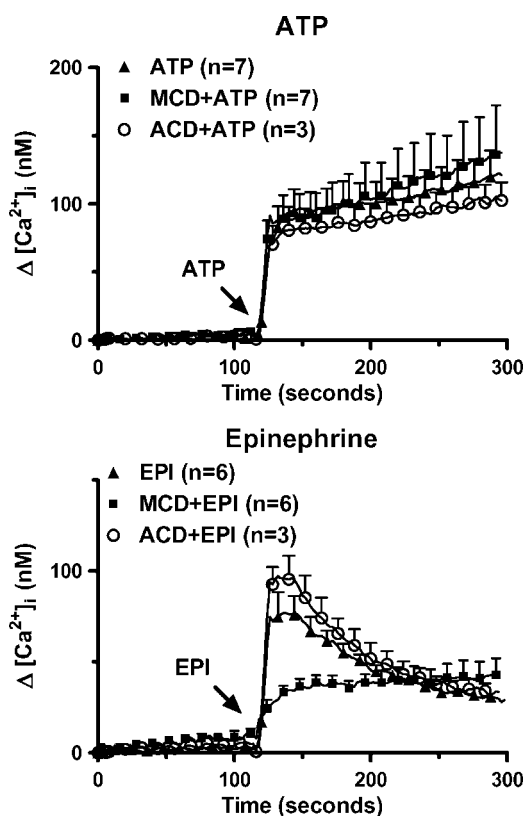

Fig. 2. Effect of MCD and α-cyclodextrin (ACD) on the variation of the intracellular concentration of calcium ($[Ca^{2+}]_i$) in cells of rat submandibular glands in response to ATP or epinephrine (EPI). A crude cellular suspension from rat submandibular gland was prepared and loaded with fura-2/AM as described in Experimental Procedures. After washing, cells were resuspended in 2 ml of HEPES-buffered saline (HBS) medium in the absence of magnesium but in the presence of 1 mM calcium. Before measurement of the $[Ca^{2+}]_i$, some cells were incubated for 10 min at 37°C in the presence of 25 mM MCD or ACD. The $[Ca^{2+}]_i$ was measured at 25°C in the same medium. Basal levels of $[Ca^{2+}]_i$ averaged 88 and 92 nM in control and ACD-treated cells, respectively, and 150 nM in MCD-treated cells. These concentrations remained stable for at least 5 min. One millimolar ATP (upper panel) or 100 μM epinephrine (lower panel) was added at 2 min (arrows). The traces were calibrated as described in Experimental Procedures. Results are expressed as variations of the $[Ca^{2+}]_i$ after addition of the agonist. Results are means $\pm$ SEM of the number of experiments indicated.
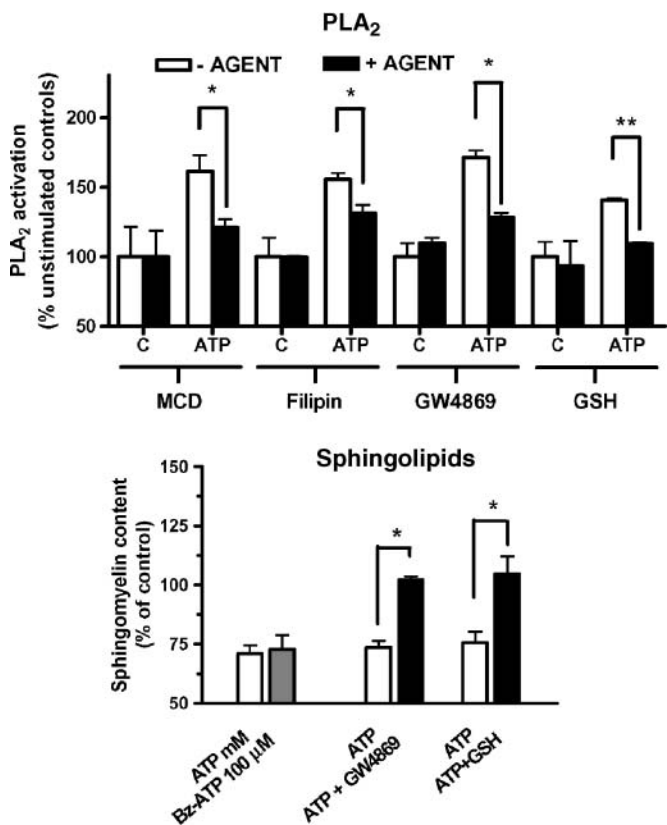

Fig. 3. Phospholipase A$_2$ (PLA$_2$) activity and level of sphingomyelin in submandibular cells exposed to ATP. Upper panel: The cells were labeled with oleic acid. They were preincubated in the presence or absence of the different agents as described in Experimental Procedures, then they were then incubated at 37°C for 10 min in control conditions or in the presence of 1 mM ATP. Results are means $\pm$ SEM of three to four experiments and are expressed as percentages of the oleic acid released in the medium compared with the corresponding control (C) (incubation in the absence of ATP). Lower panel: Cells were labeled with palmitic acid and choline. They were preincubated as described above, then they were incubated for 30 min in control conditions or in the presence of 1 mM ATP or 100 μM 2′-3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (Bz-ATP). At the end of the incubation, the lipids were extracted from the cells and sphingomyelin was measured. Results are means $\pm$ SEM of three experiments and are expressed as percentages of the sphingomyelin levels compared with the corresponding controls (incubation in the absence of ATP). * $P < 0.05$, ** $P < 0.01$ compared with the corresponding controls (incubation in the presence of ATP, in the absence of the tested agent). The effect of 1 mM ATP by itself in the absence of the tested agent was always significant ($P < 0.01$) compared with unstimulated samples.

89 ± 12 nM in MCD-treated cells, 20 s after the addition of ATP (n = 7)]. The $[Ca^{2+}]_i$ was also increased in response to epinephrine, which activates a receptor coupled to a G protein and to the hydrolysis of polyphosphoinositides. Thus, the effect of MCD on the response to 100 μM epinephrine was investigated for comparison (Fig. 2). MCD potently inhibited the response to epinephrine [from 77 ± 8 nM in control cells to 29 ± 3 nM in MCD-treated cells, 10 s after the addition of epinephrine (n = 6)]. ACD, an inactive isomer of MCD that does not disrupt rafts, had no significant effect on the variation of the $[Ca^{2+}]_i$ in response to either ATP or epinephrine (Fig. 2). Raft disruption with MCD had no significant effect on the basal activity of PLA$_2$ [from 3.7 ± 0.8% to 3.7 ± 0.7% of the oleic acid incorporated in total lipids (n = 4)] but inhibited the activation of the enzyme by ATP: ATP increased PLA$_2$ activity up to 161 ± 12% (n = 4) in control cells and up to 121 ± 6% (n = 4) in cells preincubated with MCD ($P = 0.023$) (**Fig. 3**). Similar results were obtained with cells preincubated for 30 min at 37°C with filipin. The detergent had no effect on the increase of the $[Ca^{2+}]_i$ in response to 1 mM ATP but significantly inhibited the response to epinephrine by ~40% (see supplementary **Fig. I**). Filipin inhibited the activation of PLA$_2$ by ATP [from 155 ± 4% in control cells to 131 ± 6% in filipin-treated cells (n = 3, $P = 0.022$)] (Fig. 3, upper panel).

Effect of extracellular ATP on sphingolipid metabolism

Because sphingolipids are major constituents of raft microdomains, the effect of ATP on the metabolism of these lipids was next examined. As shown in **Fig. 4A**, ATP significantly increased the ceramide content of submandibular gland cells, from 2.24 ± 0.14 to 3.51 ± 0.20 pmol ceramide/nmol phosphate (n = 8). We hypothesized that this response to ATP was most likely mediated by the P2X$_7$ receptor, and this hypothesis was tested using either Bz-ATP, a purinergic agonist with much higher affinity for P2X$_7$ receptors than ATP itself, or magnesium, which decreases the concentration of ATP^{4-} and completely blocks the response of P2X$_7$ receptors to ATP (3). One hundred micromolar Bz-ATP fully reproduced the response to ATP (Fig. 4A), which was totally blocked by the addition of 5 mM magnesium chloride (Fig. 4B). This increase in ceramide content was concomitant with a 25% decrease in sphingomyelin (n = 3, $P = 0.016$) (Fig. 4C), which was reproduced by 100 μM Bz-ATP [a 27% decrease after 30 min (n = 3, $P = 0.011$)] (Fig. 3, lower panel). At the level of plasma membranes, ATP also increased the ceramide content of rafts, from 2.66 ± 0.24 to 4.05 ± 0.20 pmol/nmol phosphate (n = 3) (Fig. 4D). The effect of ATP in the nonraft fraction was only marginal and nonsignificant [from 2.33 ± 0.01 to 2.67 ± 0.22 pmol/nmol phosphate (n = 3)].
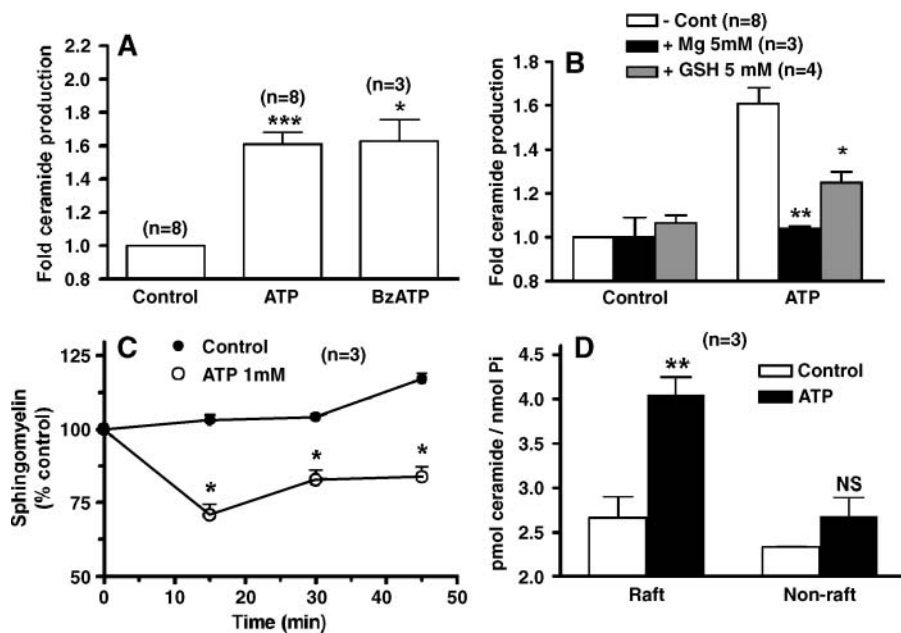

Fig. 4. Effect of ATP on the ceramide and sphingomyelin contents of submandibular cells. A: Cells from rat submandibular glands were incubated at 37°C for 30 min in the absence or presence of 1 mM ATP or 100 μM Bz-ATP. B: Cells were incubated in the absence or presence of 5 mM magnesium or were preincubated with GSH and then incubated for 30 min in the presence or absence of 1 mM ATP. At the end of the incubation, the lipids were extracted from the cells. C: Cells were labeled with palmitic acid and choline. They were incubated for various times in control conditions or in the presence of 1 mM ATP. At the end of the incubation, the lipids were extracted from the cells and sphingomyelin was measured. D: Cells were incubated in the absence or presence of 1 mM ATP. Fractions were prepared as described for Fig. 1, and the lipids were extracted. Ceramides and phosphorus were assayed as described in Experimental Procedures. Results are expressed as pmol ceramides/nmol phosphate and are means ± SEM of the number of experiments indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. NS, not significant ($P > 0.05$).

These results suggested that the increase in ceramide could be attributable to the hydrolysis of sphingomyelin rather than to the de novo synthesis of ceramides. In agreement with this hypothesis, incubation of submandibular gland cells with myriocin [an inhibitor of serine palmitoyltransferase (30)] had no effect on the cellular level of ceramides either in control conditions or in the presence of 1 mM ATP (data not shown). We next examined the effect of ATP on the sphingomyelinase activities. N-SMase and A-SMase were assayed according to Liu and Hannun (24) at pH 7.4 and 5.0, respectively. A-SMase activity was ~50-fold higher than N-SMase activity [0.96 ± 0.10 nmol SM/h/mg protein (n = 3) for N-SMase and 46 and 49 nmol SM/h/mg protein (two experiments) for A-SMase]. Interestingly, extracellular ATP had no significant effect on the activity of A-SMase (**Fig. 5**, upper panel) but increased the activity of N-SMase, by 57 ± 4% (n = 3, $P = 0.025$) (Fig. 5, lower panel). The contribution of this enzyme to the hydrolysis of cellular sphingolipids was confirmed using GSH and GW4869, which are selective inhibitors of N-SMase (28, 31). Preincubation of the cells with these inhibitors prevented the degradation of sphingomyelin (Fig. 3, lower panel). GSH also suppressed the increase of ceramide in response to ATP (Fig. 4B). Despite GSH and GW4869 preventing the hydrolysis of sphingomyelin and PLA_2 activity by ATP (Fig. 3), these inhibitors had no effect on the $[Ca^{2+}]_i$ (**Fig. 6**). This suggests that ATP increases cellular calcium concentrations by a mechanism that is independent of sphingomyelin hydrolysis and ceramide accumulation in lipid rafts.

DISCUSSION

In this work, we show that two populations of purinergic $P2X_7$ receptors are present in the plasma membrane of rat submandibular glands. After sonication of the plasma membrane, one population migrates in a sucrose gradient together with rafts; the second population migrates with most of the proteins of the membrane in fractions more dense. This distribution of $P2X_7$ receptors was greatly affected by the disruption of rafts with MCD. Rafts are microdomains of the plasma membrane containing a
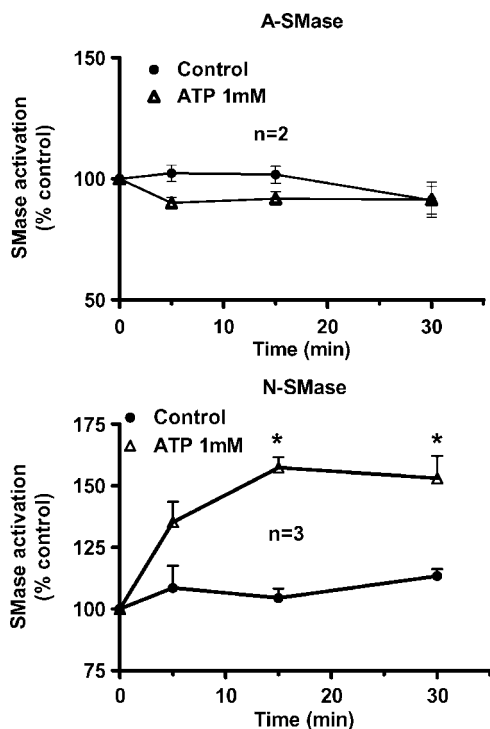

Fig. 5. Effect of ATP on the activity of the sphingomyelinases. Cells from rat submandibular glands were incubated at 37°C for various times in control conditions or in the presence of 1 mM ATP. After the extraction of the lipids, the activities of the neutral sphingomyelinase (N-SMase) and the acidic sphingomyelinase (A-SMase) were measured as described in Experimental Procedures. The specific activity of the enzyme at time 0 was used as the control, and the results are expressed as percentages of this control. Results are means with range of two experiments for A-SMase and means ± SEM of three experiments for N-SMase. * $P < 0.05$.
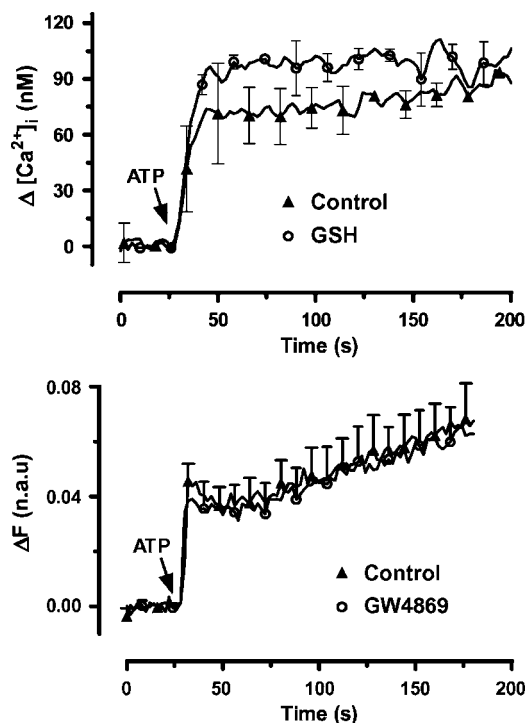

Fig. 6. Effect of N-SMase inhibitors on the variation of the $[Ca^{2+}]_i$ in cells of rat submandibular glands in response to ATP. A crude cellular suspension from rat submandibular gland was prepared and loaded with fura-2/AM (upper panel) or fluo-4/AM (lower panel) as described in Experimental Procedures. After washing, the cells were treated with the different agents as described. Assays were carried out in HBS medium in the absence of magnesium but in the presence of 1 mM calcium. Basal levels of $[Ca^{2+}]_i$ averaged 88 nM in control cells and 148 nM in GSH-treated cells. Basal calcium levels remained stable for at least 5 min. One millimolar ATP was added as indicated (arrows). Results are expressed as variations of the $[Ca^{2+}]_i$ (upper panel) or normalized arbitrary units (n.a.u.; lower panel) after the addition of ATP. Results are means with range of two experiments (upper panel) or means ± SEM of three experiments (lower panel).

high proportion of sphingolipids and cholesterol. They are also enriched in saturated fatty acids at the expense of polyunsaturated fatty acids. These domains have been compared with platforms on which proteins involved in cellular signaling are grouped. This seems to speed up cellular activation processes. Here, we report the isolation of a low-density membrane fraction with properties analogous to those of rafts. This fraction is devoid of intracellular membranes (endoplasmic reticulum or Golgi). It is only slightly contaminated by a nonraft protein (TfR) and is highly enriched in the raft markers caveolin-1 and GM1 gangliosides. Western blotting demonstrated that >50% of the $P2X_7$ receptors could be observed in rafts.

Our results are fully consistent with those of Bannas et al. (32). Using mouse lymphoma cells, they suggested that $P2X_7$ receptors were distributed among raft and nonraft domains of the plasma membrane. Two other groups have already reported on the presence of other P2X receptors in rafts. Vacca et al. (33) showed that $P2X_3$ receptors are located exclusively in rafts in the central nervous system or exogenously expressed in SH-SY5Y neuroblastoma cells. They reached this conclusion using rafts that were prepared with Triton X-100 and with a detergent-free method. Using only the method based on Triton X-100, they concluded that $P2X_1$, $P2X_2$, $P2X_4$, $P2X_7$, or $P2Y_4$ receptors were excluded from the rafts. The discrepancy between the latter results on the one hand and the results of Bannas et al. (32) and our own data on the other hand might be explained by differences in cell types or in the procedures used in the preparation of rafts. More recently, Vial and Evans (34) reported that $P2X_1$ receptors colocalize with raft markers in arterial smooth muscle. Disruption of rafts with MCD blocked purinergic-mediated currents and smooth muscle contraction.

Of importance, a major finding of this work is that ATP increases the cellular content in ceramides. This response to extracellular ATP is mediated by $P2X_7$ receptors, as it can be blocked by extracellular magnesium and reproduced by a relatively low concentration (100 μM) of Bz-ATP. At the membrane level, ATP also significantly increased the ceramide content in rafts but not in the nonraft fractions. Such an increase might contribute to the formation of platforms on which molecules involved in signal transduction accumulate. ATP could also trigger
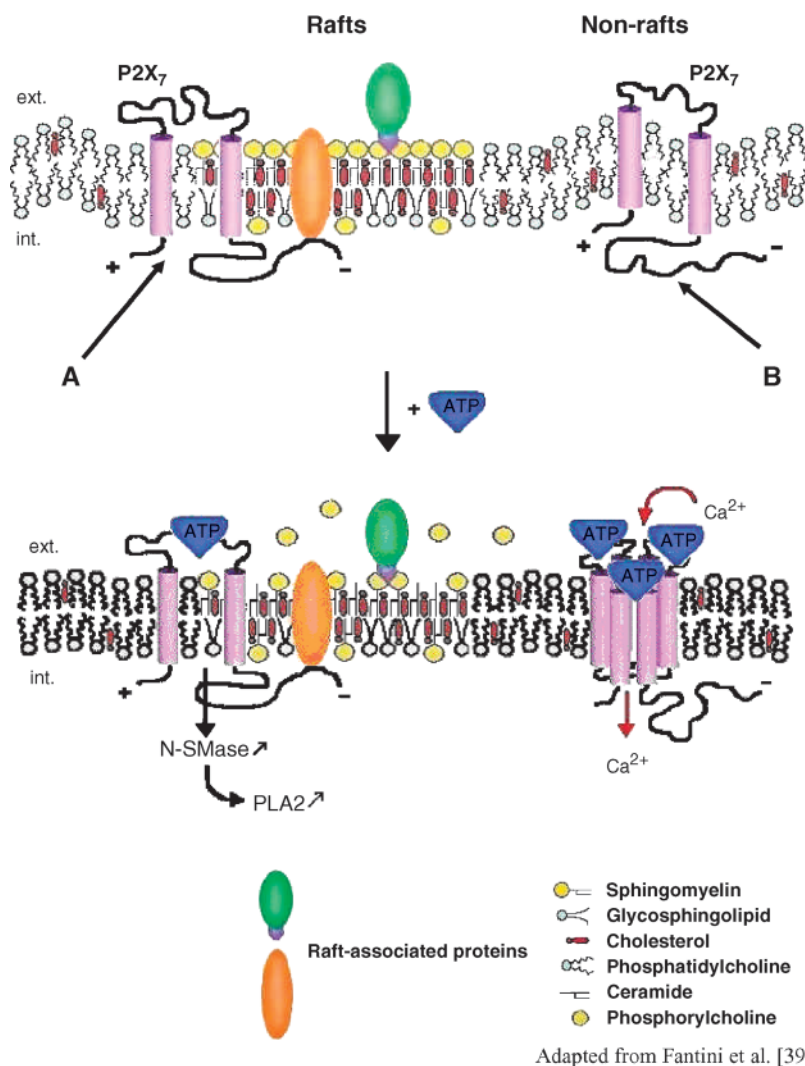

Fig. 7. Model summarizing the role of the two populations of $P2X_7$ receptors in rat submandibular gland. Upper panel: The plasma membrane is heterogeneous with sphingolipid- and cholesterol-rich microdomains (rafts) dispersed in a fluid phase containing mainly glycerophospholipids. A fraction of $P2X_7$ receptors interacts with a protein located in rafts (A). Lower panel: The activation of the receptors triggers two distinct responses. The receptors located in the nonraft fraction (B) are associated with the formation of a nonselective cation channel. The receptors associated with the rafts (A) are coupled with the activation of N-SMase and the release of phosphorylcholine and ceramides from the sphingolipids of the rafts. PLA_2 is activated in response to the stimulation of N-SMase. These models were adapted from Fantini et al. (39). Ext, extracellular; int, intracellular.

the blebbing and vesiculation of the membrane, which are signs of apoptosis (13). Indeed, our results are reminiscent of those reported for TNFR1. The activation of this receptor is also coupled to an increased content of ceramides (35) that is secondary to the activation of sphingomyelinases. Considering that myriocin, an inhibitor of the rate-limiting step for ceramide synthesis (30), did not block the increase of ceramide in response to ATP and that the increase of ceramide was accompanied by a decrease in sphingomyelin levels, it was hypothesized that ATP might increase sphingomyelinase activity. Therefore, both N- and A-SMase activities were measured in submandibular gland cells. We observed that the activity of A-SMase was much higher than the activity of N-SMase, in agreement with previous work (36). Interestingly, ATP selectively increased the activity of N-SMase, an enzyme that has been located in rafts (36). It is thus tempting to speculate that the occupancy of the P2X$_7$ receptors present in rafts activates N-SMase and that this enzyme activity is responsible for the increased content of ceramides in these microdomains. The role of ceramides in cellular function was next considered. This lipid is a mediator of cell death induced by TNF or other cytokines and might be a mediator of the apoptotic response to ATP and TNF (37). The inhibition of N-SMase with GSH or GW4869 blocked the activation of PLA$_2$ in response to ATP. These results suggest that this response is secondary to the activation of N-SMase and are consistent with the results of Wiegmann et al. (38), who reported that the activation of N-SMase by TNFR1 was responsible for the secondary activation of PLA$_2$.

The physiological role of the P2X$_7$ receptors that are not located in rafts was also examined. To this end, the cells were preincubated with MCD. This molecule extracts cholesterol from the plasma membrane and disrupts the organization of rafts. MCD almost completely abolished the increase of the $[Ca^{2+}]_i$ caused by epinephrine. This is in agreement with our previous observations (22) and is best explained by the presence in rafts of the transducing protein coupling the adrenergic receptor to phospholipase C. These results confirmed the effectiveness of the treatment with MCD on the disorganization of the rafts. Similar results were obtained with filipin, a molecule that disrupts lipid rafts through its interaction with cholesterol. MCD and filipin differently affected various responses to extracellular ATP. In response to specific agonists, P2X$_7$ receptors rapidly form a nonselective cation channel that is permeant not only to monovalent cations but also to calcium or manganese (3). The uptake of calcium through this nonselective channel accounts for the increase of $[Ca^{2+}]_i$ in response to ATP. MCD or filipin and GSH or GW4869 had no effect on this increase of $[Ca^{2+}]_i$, suggesting that the nonselective cation channel is formed by the population of the receptors not located in rafts and that its formation is independent of the activation of N-SMase. By contrast, MCD or filipin and GSH or GW4869 inhibited the ATP-activated PLA$_2$. The location of this enzyme in rafts might facilitate its inhibition. However, the lack of inhibition by MCD of the basal activity of PLA$_2$ does not support this hypothesis. An alternative explanation consistent with the results discussed above would be that raft disruption interferes with the activation process of the P2X$_7$ receptors that are present in these microdomains.

In conclusion, we have established the presence of two populations of P2X$_7$ receptors in the plasma membrane of submandibular gland cells (**Fig. 7**). The receptors present in nonraft microdomains are involved in the formation of a nonselective cation channel that is independent of sphingomyelin hydrolysis and PLA$_2$ activation. The receptors present in rafts regulate the activity of an N-SMase, initiating a cascade leading to PLA$_2$ activation.∎

REFERENCES

1. Turner, J. T., L. A. Landon, S. J. Gibbons, and B. R. Talamo. 1999. Salivary gland P2 nucleotide receptors. *Crit. Rev. Oral Biol. Med.* **10:** 210–224.
2. Burnstock, G. 2004. Introduction: P2 receptors. *Curr. Top. Med. Chem.* **4:** 793–803.
3. North, R. A. 2002. Molecular physiology of P2X receptors. *Physiol. Rev.* **82:** 1013–1067.
4. Ennion, S. J., and R. J. Evans. 2002. Conserved cysteine residues in the extracellular loop of the human P2X$_1$ receptor form disulfide bonds and are involved in receptor trafficking to the cell surface. *Mol. Pharmacol.* **61:** 303–311.
5. Pochet, S., A. Gomez-Munoz, A. Marino, and J. P. Dehaye. 2003. Regulation of phospholipase D by P2X$_7$ receptors in submandibular ductal cells. *Cell. Signal.* **15:** 927–935.
6. Surprenant, A., F. Rassendren, E. Kawashima, R. A. North, and G. Buell. 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X$_7$). *Science.* **272:** 735–738.
7. Smart, M. L., B. Gu, R. G. Panchal, J. Wiley, B. Cromer, D. A. Williams, and S. Petrou. 2003. P2X$_7$ receptor cell surface expression and cytolytic pore formation are regulated by a distal C-terminal region. *J. Biol. Chem.* **278:** 8853–8860.
8. Virginio, C., A. MacKenzie, R. A. North, and A. Surprenant. 1999. Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X$_7$ receptor. *J. Physiol.* **519:** 335–346.
9. Chow, S. C., C. E. Kass, and S. Orrenius. 1997. Purines and their roles in apoptosis. *Neuropharmacology.* **36:** 1149–1156.
10. Tsukimoto, M., H. Harada, A. Ikari, and K. Takagi. 2005. Involvement of chloride in apoptotic cell death induced by activation of ATP-sensitive P2X$_7$ purinoceptor. *J. Biol. Chem.* **280:** 2653–2658.
11. Kahlenberg, J. M., and G. R. Dubyak. 2004. Mechanisms of caspase-1 activation by P2X$_7$ receptor-mediated K$^+$ release. *Am. J. Physiol. Cell Physiol.* **286:** C1100–C1108.
12. Le Stunff, H., R. Auger, J. Kanellopoulos, and M. N. Raymond. 2004. The Pro-451 to Leu polymorphism within the C-terminal tail of P2X$_7$ receptor impairs cell death but not phospholipase D activation in murine thymocytes. *J. Biol. Chem.* **279:** 16918–16926.

13. Wilson, H. L., S. A. Wilson, A. Surprenant, and R. A. North. 2002. Epithelial membrane proteins induce membrane blebbing and interact with the P2X$_7$ receptor C terminus. *J. Biol. Chem.* **277:** 34017–34023.

14. Denlinger, L. C., P. L. Fisette, J. A. Sommer, J. J. Watters, U. Prabhu, G. R. Dubyak, R. A. Proctor, and P. J. Bertics. 2001. Cutting edge. The nucleotide receptor P2X$_7$ contains multiple protein- and lipid-interaction motifs including a potential binding site for bacterial lipopolysaccharide. *J. Immunol.* **167:** 1871–1876.

15. Weber, C. H., and C. Vincenz. 2001. The death domain superfamily: a tale of two interfaces? *Trends Biochem. Sci.* **26:** 475–481.

16. Cottin, V., J. E. Doan, and D. W. Riches. 2002. Restricted localization of the TNF receptor CD120a to lipid rafts: a novel role for the death domain. *J. Immunol.* **168:** 4095–4102.

17. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* **387:** 569–572.

18. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science.* **175:** 720–731.

19. Garcia-Marcos, M., S. Pochet, S. Tandel, M. Villate, F. Gomez, A. Marino, and J. P. Dehaye. 2004. Insensitivity to raft disruption of SOCC and purinergic coupled NSCC in rat submandibular glands. 4th International Symposium of Nucleosides and Nucleotides in Chapel Hill, NC, June 6–9, 2004.

20. Amsallem, H., M. Métioui, A. VandenAbeele, A. Elyamani, A. Moran, and J. P. Dehaye. 1996. Presence of a metabotropic and an ionotropic purinergic receptor on rat submandibular ductal cells. *Am. J. Physiol.* **271:** C1546–C1555.

21. Liu, Y., L. Casey, and L. J. Pike. 1998. Compartmentalization of phosphatidylinositol 4,5-bisphosphate in low-density membrane domains in the absence of caveolin. *Biochem. Biophys. Res. Commun.* **245:** 684–690.

22. Garcia-Marcos, M., S. Tandel, S. Pochet, J. Genin, F. Gomez, A. Kumps, A. Marino, and J. P. Dehaye. 2005. Cholesterol depletion perturbs calcium handling by rat submandibular glands. *J. Cell. Physiol.* **203:** 429–438.

23. Alzola, E., A. Perez-Etxebarria, E. Kabré, D. J. Fogarty, M. Métioui, N. Chab, J. M. Macarulla, C. Matute, J. P. Dehaye, and A. Marino. 1998. Activation by P2X$_7$ agonists of two phospholipases A$_2$ in ductal cells of rat submandibular gland. Coupling of the iPLA$_2$ with kallikrein secretion. *J. Biol. Chem.* **273:** 30208–30217.

24. Liu, B., and Y. A. Hannun. 2000. Sphingomyelinase assay using radiolabeled substrate. *Methods Enzymol.* **311:** 164–167.

25. Bligh, E. F., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.

26. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorous. *J. Biol. Chem.* **66:** 375–400.

27. Perry, D. K., A. Bielawska, and Y. A. Hannun. 2000. Quantitative determination of ceramide using diglyceride kinase. *Methods Enzymol.* **312:** 22–31.

28. Luberto, C., D. F. Hassler, P. Signorelli, Y. Okamoto, H. Sawai, E. Boros, D. J. Hazen-Martin, L. M. Obeid, Y. A. Hannun, and G. K. Smith. 2002. Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. *J. Biol. Chem.* **277:** 41128–41139.

29. Macdonald, J. L., and L. J. Pike. 2005. A simplified method for the preparation of detergent-free lipid rafts. *J. Lipid Res.* **46:** 1061–1067.

30. Erdreich-Epstein, A., L. B. Tran, O. T. Cox, E. Huang, W. E. Laug, H. Shimada, and M. Millard. 2005. Endothelial apoptosis induced by inhibition of integrins alphavbeta3 and alphavbeta5 involves ceramide metabolic pathways. *Blood.* **105:** 4353–4361.

31. Liu, B., N. Andrieu-Abadie, T. Levade, P. Zhang, L. M. Obeid, and Y. A. Hannun. 1998. Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death. *J. Biol. Chem.* **273:** 11313–11320.

32. Bannas, P., S. Adriouch, S. Kahl, F. Braasch, F. Haag, and F. Koch-Nolte. 2005. Activity and specificity of toxin-related mouse T cell ecto-ADP-ribosyltransferase ART2.2 depends on its association with lipid rafts. *Blood.* **105:** 3663–3670.

33. Vacca, F., S. Amadio, G. Sancesario, G. Bernardi, and C. Volonte. 2004. P2X$_3$ receptor localizes into lipid rafts in neuronal cells. *J. Neurosci. Res.* **76:** 653–661.

34. Vial, C., and R. J. Evans. 2005. Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. *J. Biol. Chem.* **280:** 30705–30711.

35. Xu, J., C. H. Yeh, S. Chen, L. He, S. L. Sensi, L. M. Canzoniero, D. W. Choi, and C. Y. Hsu. 1998. Involvement of de novo ceramide biosynthesis in tumor necrosis factor-alpha/cycloheximide-induced cerebral endothelial cell death. *J. Biol. Chem.* **273:** 16521–16526.

36. Veldman, R. J., N. Maestre, O. M. Aduib, J. A. Medin, R. Salvayre, and T. Levade. 2001. A neutral sphingomyelinase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain: potential implications in tumour necrosis factor signalling. *Biochem. J.* **355:** 859–868.

37. Gulbins, E., and R. Kolesnick. 2003. Raft ceramide in molecular medicine. *Oncogene.* **22:** 7070–7077.

38. Wiegmann, K., S. Schutze, T. Machleidt, D. Witte, and M. Kronke. 1994. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell.* **78:** 1005–1015.

39. Fantini, J., N. Garmy, R. Mahfoud, and N. Yahi. 2002. Lipid rafts: structure, function and role in HIV, Alzheimers and prion diseases. *Expert Rev. Mol. Med.* **4:** 1–22.