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Bromoenol lactone enhances the permeabilization of rat submandibular acinar cells by $P2X_7$ agonists

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1 The permeabilizing effect of $P2X_7$ agonists was tested in rat submandibular acinar cells using the uptake of ethidium bromide as an index.

2 The uptake of ethidium bromide by acini incubated at 37°C in the presence of 1 mM ATP increased with time and reached after 5 min about 10% of maximal uptake measured in the presence of digitonin.

3 The response to ATP was dose-dependent (half-maximal concentration around 40 μM) and it was decreased when the temperature was lowered to 25°C.

4 Benzoyl-ATP reproduced the response to ATP (half-maximal concentration around 10 μM). UTP or 2-methylthioATP had no effect.

5 The permeabilization in response to ATP was blocked by oxidized ATP and by magnesium and inhibited by Coomassie blue.

6 ATP increased the activity of a calcium-insensitive phospholipase A_2 ($iPLA_2$).

7 Bromoenol lactone (BEL) inhibited the $iPLA_2$ stimulated by ATP but potentiated the uptake of ethidium bromide in response to the purinergic agonist.

8 From these results it is concluded that the activation of $P2X_7$ receptors permeabilizes rat submandibular acinar cells. The pore-forming activity of the receptor might be negatively regulated by the concomitant activation of the $iPLA_2$ by the receptor.
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Abbreviations: Bz-ATP, 2′ & 3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; fura-2/AM, fura 2-acetoxymethyl ester; HBS, HEPES buffered saline; 2-MeSATP, 2-methylthioadenosinetriphosphate; oATP, adenosine 5′-triphosphate-2′,3′ dialdehyde; RT–PCR, reverse transcription-polymerase chain reaction

Introduction

The existence of receptors mediating the effect of extracellular nucleotides on cell function was first proposed by Burnstock in 1971. The numerous purinergic receptors which were subsequently characterized have now been classified in two major groups. P2Y receptors have seven transmembrane domains and belong to the superfamily of receptors coupled to G proteins. Purinergic receptors of the second group (the P2X receptors) are cation-selective channels gated by extracellular ATP. These proteins have only two transmembrane domains, a large extracellular cysteine-rich (ten residues) loop and intracellular amino and carboxy terminals (Soto *et al*., 1997). Seven P2X subunits have been cloned. They assemble as homomers or heteromers (Lewis *et al*., 1995). The $P2X_7$ is the most structurally different (less than 25% identity) and has a C-terminal domain which is 200 amino acids longer than the other P2X receptors (Surprenant *et al*., 1996). It has also unique properties: (1) it is activated by high concentrations of ATP; (2)/ 2′ and 3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (Bz-ATP) is the best agonist; (3) removal of extracellular magnesium strongly potentiates the response to ATP and (4) exposure to an agonist for a long time opens a transmembrane pore with a diameter of several nanometers which is permeant to large molecules like propidium dyes or ethidium bromide (Nuttle & Dubyak, 1994). The cytolytic effect of $P2X_7$ receptors has identified them as the P_{2Z} receptors previously described in macrophages, microglial, or immune cells (El-Moatassim & Dubyak, 1992; Ferrari *et al*., 1997; Coutinho-Silva & Persechini, 1997). Truncated $P2X_7$ receptors lacking the C-terminal end do not exhibit this permeabilizing effect and it is suspected that the association of subunits to a multimeric structure progressively increases the size of the channel formed in the membrane.

The responsiveness of salivary glands to extracellular ATP was first observed by Gallacher in 1982. The secretion of amylase and the permeability to ions of the plasma membrane were increased when mouse parotid acini were exposed to ATP. Soltoff *et al*. (1992) reported that rat parotid acini also responded to extracellular ATP, which opened a non-specific cation channel. The tetrabasic form of ATP activated a purinergic receptor responsible for a massive increase of $[Ca^{2+}]_i$ and $[Na^+]_i$, and for the depolarization of the plasma membrane (Soltoff *et al*., 1992; Hurley *et al*., 1996). Bz-ATP was an even better agonist than ATP^{4-}. But the addition of these agonists to the incubation medium did not permeabilize the cells (Soltoff *et al*., 1992). This result suggested that ATP bound to a P2X receptor other than a $P2X_7$ receptor. Indeed Buell *et al*. (1996) showed that $P2X_4$ receptors were expressed in acini from rat submandibular gland. Yet Collo *et al*. (1997) reported that $P2X_7$ receptors were expressed in rat submandibular glands. Using reverse transcription-polymerase chain reaction (RT–PCR), we have recently observed transcripts for $rP2X_7$ not only in a pure ductal preparation from rat submandibular gland but also in acini (Alzola *et al*.,

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1998). Similar results have been reported by Lee *et al*. (1997) and by Tenneti *et al*. (1998). The purpose of this work was to test the permeabilizing effect of extracellular nucleotides on a pure acinar suspension. The second goal of this work was to study the mechanisms regulating the pore-forming activity of the receptor.

Methods

Materials

Male Wistar rats (200–250 g) were purchased from the Proefdierencentrum of the Katholieke Universiteit Leuven (Heverlee, Belgium). The animals were fed ad libitum and had free access to water. The care and use of rats in this study was approved by the Belgian Ministry of Agriculture in agreement with EEC regulations.

Collagenase P, bovine serum albumin (BSA, fraction V), adenosine 5′-triphosphate (ATP), ethylene glycol-bis(β-amino ethylether)-N,N,N′,N′-tetraacetic acid (EGTA), and 4-(2-hydroxyethyl) 1-piperazine-ethane-sulphonic acid (HEPES) were from Boehringer (Mannheim, Germany). Digitonin, carbamylcholine, 2′ and 3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (Bz-ATP), adenosine 5′-triphosphate -2′,3′ dialdehyde (oATP) and isoproterenol bitartrate were supplied by Sigma Chemical Company (St Louis, MO, U.S.A.). The pluronic acid and the fura 2-acetoxymethyl ester (Fura-2/AM) were from Molecular Probes (Eugene, OR, U.S.A.). The amino acid mixture (without glutamine) was from Gibco (Uxbridge, U.K.). Coomassie blue was from Aldrich Chimie (Milwaukee, WI, U.S.A.), arachidonyl trifluoromethylketone ($AACOCF_3$) from Alexis Biochemicals (Woburn, MA, U.S.A.), 2-methylthioadenosine-triphosphate (2-MeSATP) from Research Biochemical International (Natick, MA, U.S.A.). (E)-6-(bromoethylene)tetrahydro-3-(1-naphtalenyl-2H-pyron-2-one (bromoenol lactone, BEL) was purchased from Cayman Scientific (Beneendael, The Netherlands). The 9,10-^{3}H(N) oleic acid was provided by ARC (St Louis MO, U.S.A.).

Isolation of rat submandibular acini and ducts

The rats were anaesthetized and killed by exsanguination. The submandibular glands were excised and dissected. The glands were finely minced in 0.3 ml of HEPES buffered saline (HBS) containing (mM): NaCl 96, KCl 6, $MgCl_2$ 1, NaH_2PO_4 2.5, glucose 11, Na-pyruvate 5, Na-glutamate 5, Na-fumarate 5, HEPES 24.5 (pH 7.4 with NaOH), amino acid mixture 0.1% (w v^{-1}). The minced tissue was then incubated for 20 min under constant shaking (160 cycles min^{-1}) at 37°C in 10 ml of the same medium but containing 1.4 mg collagenase P (2.6 U mg^{-1}). After the first 10 min of incubation, the tissue was aspirated 10 times in a 10 ml glass pipet. At the end of the incubation, the suspension was aspirated through a 10 ml, then a 5 ml and a 2 ml pipet. This crude suspension was washed four times with the HBS medium. The final pellet was resuspended in 6 ml of this medium. Two 15 ml tubes were filled with 6 ml of 40% isotonic Percoll. Three ml of the crude cellular suspension were layered on top of the Percoll and the tubes were centrifuged at 4°C for 10 min at $4000 \times g$. At the end of the centrifugation, two bands could be distinguished: an upper band on the top of the Percoll contained the ducts while the acini had sedimented (Amsallem *et al*., 1996). The band containing the acini was aspirated and washed three times with 10 ml isotonic saline. After the last wash, the pellet was resuspended in HBS medium and kept at 4°C until use.

Measurement of the plasma membrane permeability

The permeabilization of the plasma membrane was measured with the fluorescent dye ethidium bromide (Di Virgilio *et al*., 1989). The cells were washed and resuspended in HBS medium in the absence of calcium and magnesium. The cell suspension was incubated in the cuvette of the fluorimeter at 37°C and constantly stirred. Ethidium bromide was added to a final concentration of 20 μM. Cells were allowed to equilibrate for 10 min before the start of the measurement. The excitation wavelength was 360 nm (slitwidth 4 nm). The light emitted at 580 nm (slitwidth 16 nm) was measured each second. The maximum uptake of ethidium was estimated by adding digitonin (20 μg ml^{-1}) in the cuvette. The permeabilizing effect of various agonists was estimated by comparison with the permeabilization measured in the presence of digitonin.

Measurement of the release of [^{3}H]-oleic acid

The cellular suspension was resuspended in 1 ml HBS medium in the presence of 0.5 mM $CaCl_2$. Three μCi ml^{-1} [^{3}H]-oleic acid were added and the suspension was incubated for 1 h at 25°C with gentle shaking. At the end of the labelling period, the cells were washed and incubated for 1 h in 6 ml calcium-free HBS medium in the absence of the tracer. After this incubation, the cells were washed again and resuspended in 6 ml calcium-free HBS medium. The assays were performed at 37°C under constant shaking and started by the addition of 100 μl of the cellular suspension to 400 μl HBS medium containing $CaCl_2$ (final concentration 0.5 mM). The incubation was stopped by centrifugation for 30 s at $10{,}000 \times g$. Four hundred μl of the supernatants were transferred to vials and mixed with 4 ml scintillation cocktail (Ecoscint® A, National Diagnostics, Atlanta, GA, U.S.A.). Radioactivity of the samples was measured in a scintillation spectrometer (1211, Minibeta, LKB Wallac). In order to estimate the radioactivity already present in the medium at the start of the assay, several samples of the cellular suspension were taken during the experiments, mixed with 400 μl HBS medium and directly centrifuged and the radioactivity present in the supernatants was counted. These results were used as blank values to estimate the release of [^{3}H]-oleic acid during the incubation at 37°C.

Statistical analysis

The effects of various conditions tested at different days were compared using an F test for the analysis of variance. The significance of the variation with time of a parameter in a batch of cells was tested using a paired *t*-test. The dependence on temperature and on the presence of ATP in Figure 1 was assessed by 1 and 2 way ANOVA test.

Results

Effect of the temperature on the permeabilization

When the cells were incubated at 37°C, 1 mM ATP steadily increased the uptake of ethidium bromide to $10.6 \pm 0.8\%$ ($P < 0.001$, $n = 16$) 5 min after the addition of the purinergic agonist (Figure 1). At 25°C, ATP was much less effective and increased the uptake of ethidium bromide to $4.8 \pm 0.3\%$

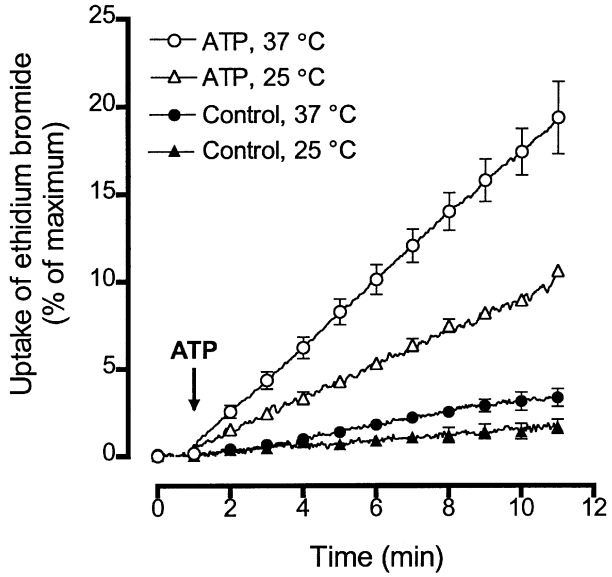

Figure 1 Effect of temperature on the uptake of ethidium bromide by rat submandibular acinar cells in response to extracellular ATP. Rat submandibular acinar cells were resuspended in a calcium-free medium and preincubated for 10 min in the cuvette of a fluorimeter at 25°C or 37°C in the presence of 20 μM ethidium bromide. They were constantly stirred. After this preincubation, the fluorescence was recorded. One minute after the start of the measurement, 1 mM ATP was added to the cuvette. Ten minutes later, the cells were permeabilized with 20 μg ml^{-1} digitonin. The results are expressed as per cent of the fluorescence measured 2 min after the addition of digitonin to the medium. They are the means$\pm$s.e.mean of three (25°C) and 16 (37°C) experiments.

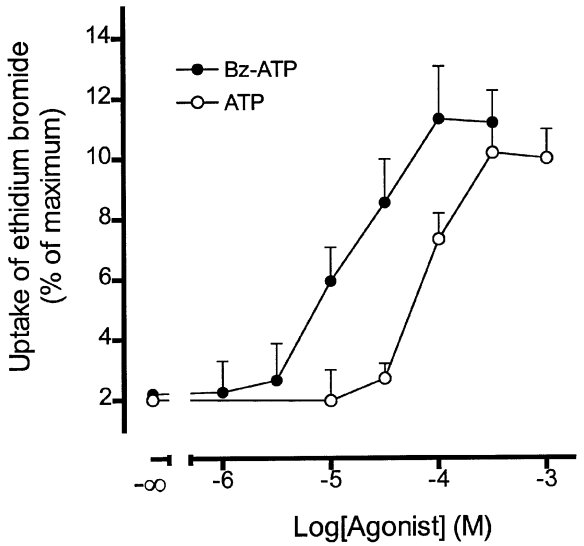

Figure 2 Comparison of the dose-response curves for ATP and Bz-ATP on the uptake of ethidium bromide by rat submandibular acinar cells. Rat submandibular acinar cells were resuspended in a calcium-free medium and preincubated for 10 min in the cuvette of a fluorimeter at 37°C in the presence of 20 μM ethidium bromide. They were constantly stirred. After this preincubation, the fluorescence was recorded. One minute after the start of the measurement, various concentrations of ATP or Bz-ATP were added to the cuvette. Ten minutes later, the cells were permeabilized with 20 μg ml^{-1} digitonin. The results were calculated using the fluorescence measured 5 min after the addition of the purinergic agonist as per cent of the fluorescence measured 2 min after the addition of digitonin to the medium. They are the means$\pm$s.e.mean of three (ATP) and eight (Bz-ATP) experiments.

($P<0.001$, $n=3$). The temperature- and time-dependence of ATP-induced uptake of ethidium bromide were assessed by ANOVA test between the curves ($P<0.001$). By comparison, 100 μM carbachol (which increases the $[Ca^{2+}]_i$) and 10 μM isoproterenol (which increases the intracellular concentration of cyclic AMP) had no effect on the uptake of ethidium bromide (data not shown).

Characterization of the purinergic receptors involved in the uptake of ethidium bromide

After a 5 min exposure to 100 μM Bz-ATP, the uptake of ethidium bromide averaged 11.3$\pm$1.8% ($n=8$), a value not significantly different from the response to 300 μM ATP. Half-maximal concentrations for ATP and Bz-ATP were 47 and 9 μM respectively (Figure 2). One mM UTP (an agonist of $P2Y_2$ receptors) and 100 μM 2-Me SATP (an agonist of $P2Y_1$ receptors) had no effect on the uptake of ethidium bromide (data not shown). In the presence of 5 mM magnesium the response to ATP significantly decreased to 31.1$\pm$4.4% of control ($n=4$; $P<0.025$). Coomassie blue (100 μM), an inhibitor of P2 purinergic receptor (Soltoff *et al*., 1989), significantly inhibited the permeabilizing effect of ATP to 40.9$\pm$6.4% of control ($n=4$; $P<0.05$). Murgia *et al*. (1993) reported that $P2X_7$ receptors were blocked by a preincubation with oATP. Preincubation for 2 h at 25°C in the presence of 100 μM oATP markedly inhibited the uptake of ethidium bromide in response to 1 mM ATP to 5$\pm$2% ($n=4$). This absence of response to the purinergic agonist was not secondary to a deleterious effect of the preincubation since the $[Ca^{2+}]_i$ of these cells increased in response to carbachol (not shown).

Effect of the inhibition of a phospholipase A_2 on the permeabilization induced by ATP

Our results suggested that the uptake of ethidium bromide in response to ATP involved $P2X_7$ receptors. We have reported that $P2X_7$ agonists can activate a calcium-sensitive phospholipase A_2 ($cPLA_2$) in ducts and a calcium-insensitive PLA_2 ($iPLA_2$) both in ducts and acini (Kabré *et al*., 1999). The role of this latter enzyme in the permeabilizing effect of ATP was evaluated with two inhibitors. In a first step, the inhibitory effect of the drugs on the PLA_2 activity of the acini was tested. The phospholipids of the acini were labelled with [^{3}H]-oleic acid. The cells were then exposed for 30 min at 25°C to 30 μM BEL, an inhibitor of the $iPLA_2$ (Hazen *et al*., 1991), or to 100 μM $AACOCF_3$, an inhibitor of the $cPLA_2$ (Street *et al*., 1993). After this preincubation, the cells were stimulated with 1 mM ATP at 37°C. In the presence of 1 mM extracellular calcium, the purinergic agonist increased by 34$\pm$6% ($n=3$) the release of [^{3}H]-oleic acid. The removal of calcium and the presence of EGTA in the medium did not affect the response to ATP (32$\pm$4%, $n=3$; $P>0.05$). This result confirmed that a $iPLA_2$ was involved in the release of [^{3}H]-oleic acid in response to ATP. In agreement with this conclusion, the preincubation with $AACOCF_3$ had no effect on the response to ATP in the absence of extracellular calcium (23$\pm$9%, $n=3$; $P>0.1$ when compared to ATP alone). The response to ATP was completely blocked by BEL (-3 ± 9%, $n=3$; $P<0.01$ when compared to ATP alone) (Table 1). The two inhibitors were then tested on the uptake of ethidium bromide (Table 1). By themselves, they had no effect on the uptake of ethidium bromide (data not shown). When the cells were preincubated with BEL, their response to extracellular ATP was not affected by BEL during the first minute stimulation. After this lag phase, the uptake of ethidium

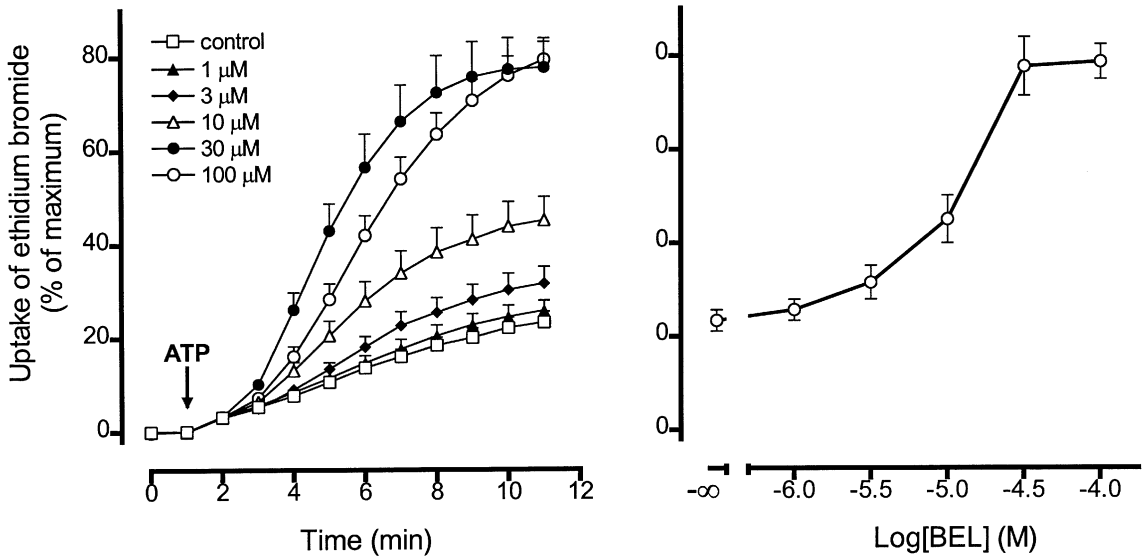

Figure 3 Effect of various concentrations of BEL on the uptake of ethidium bromide by rat submandibular acinar cells in response to ATP. Rat submandibular acinar cells were resuspended in a calcium-free medium. They were preincubated for 30 min at 25°C in the presence of various concentrations of BEL. At the end of the preincubation, they were transferred in the cuvette of a fluorimeter at 37°C in the presence of 20 μM ethidium bromide. They were constantly stirred. One minute after the start of the measurement, 1 mM ATP was added to the medium. Ten minutes later, the cells were permeabilized with 20 μg ml^{-1} digitonin. The left panel shows the time course traces and the right panel represents the corresponding values measured after 10 min incubation with 1 mM ATP. The results are expressed as per cent of maximal uptake of ethidium bromide. They are the means ± s.e.mean of six experiments.

Table 1 Effect of BEL and $AACOCF_3$ on ATP-stimulated PLA_2 activity and uptake of ethidium bromide

	Oleic acid release (% of control)		*Ethidium*
	+ Calcium 1mM	*+ EGTA* 0.5 nM	*bromide uptake* (% F_{max})
ATP	134 ± 6	132 ± 4	18 ± 2
ATP + BEL	95 ± 7	97 ± 6	78 ± 6
ATP + $AACOCF_3$	121 ± 8	123 ± 9	20 ± 3

PLA_2 activity: [^{3}H]-oleic acid labelled acinar cells were incubated for 30 min at 25°C in the presence of 30 μM BEL or $AACOCF_3$ in the stated conditions. Then they were stimulated with 1 mM ATP for 20 min at 37°C. The results are expressed as the per cent of the release of OA in control conditions (absence of ATP and of inhibitor). They are the means ± s.e.mean of three experiments. Uptake of ethidium bromide: Rat submandibular cells were preincubated as described above. Then they were incubated at 37° in the presence of 20 μM ethidium bromide for 10 min and then stimulated with 1 mM ATP for 10 min. The results are expressed as percentage of maximal uptake of ethidium bromide. they are the means ± s.e.mean of three to six experiments.

bromide sharply increased: after 10 min exposure to ATP, the uptake of ethidium bromide increased from 23.3 ± 2.3% (n = 6) to 77.6 ± 6.2% (n = 6). This potentiating effect of BEL required a preincubation with the inhibitor; it involved the $P2X_7$ receptor since it was completely blocked by magnesium (data not shown). As shown in Figure 3, the response to BEL was dose-dependent in the 1 – 100 μM concentration range (half-maximal concentration: 12 ± 2 μM). At the opposite of BEL, a preincubation with $AACOCF_3$ had no effect on the response to ATP (22.1 ± 2.8%, n = 3; $P > 0.05$) (Table 1).

Discussion

In this work we showed that extracellular ATP permeabilized acinar cells from rat submandibular glands. The permeabilization was estimated by measuring the uptake of ethidium bromide. Indeed, it has been previously shown that in response to extracellular ATP, some plasma membranes become permeant to molecules with a molecular weight up to 800 (Nuttle & Dubyak, 1994). This explains why ethidium (M_r 314) can permeate the pore and reach the nucleus where it binds nucleic acid resulting in increased fluorescence (Di Virgilio *et al*., 1989). The permeabilization in response to extracellular ATP was temperature-dependent: it was clearly seen at 37°C but not at 25°C. Such a sensitivity of the permeabilization towards temperature has been generally described (Nuttle & Dubyak, 1994). Soltoff *et al*. (1992) reported the presence of purinergic receptors coupled to a non-selective cation channel in rat parotid acini but failed to see any release of fura-2 (M_r 836) even when the cells were incubated at 37°C. We confirmed their results (data not shown) and conclude that the pores formed by $P2X_7$ receptors in salivary glands are permeant only to molecules with a molecular weight < 800.

The expression of $P2X_7$ receptors has been reported both in submandibular acini (Tenneti *et al*., 1998) and ducts (Alzola *et al*., 1998). The activation of these receptors by ATP probably accounts for the uptake of ethidium bromide observed in the presence of the purinergic agonist. Indeed, the response to ATP was not reproduced by a $P2Y_1$ agonist (2-MeSATP) or by a $P2Y_2$ agonist (UTP) but was mimicked by Bz-ATP. Bz-ATP was even more potent than ATP itself: its half-maximal concentration was around 10 μM while 40 μM ATP were required to see a half-maximal effect. The role of the $P2X_7$ receptor in the response to ATP was further confirmed by the fact that extracellular magnesium inhibited the response to ATP. This inhibition can be explained either by a decrease of the concentration of the free form of ATP or by a direct interaction of the divalent cation at the receptor level (Virginio *et al*., 1997).

The pore-forming capacity of the $P2X_7$ receptors differs between species. $P2X_7$ receptors form pores permeant to larger molecules in mice when compared to rats (Humphreys *et al*., 1998). Differences also exist between tissues. It has been

recently claimed that $P2X_7$ receptors did not form pores in human skin fibroblasts (Solini *et al*., 1999). The permeabilizing effect of ATP which we observed in rat submandibular acinar cells was much smaller than the effect of ATP on other cell types like macrophages or glial cells (Coutinho-Silva & Persechini, 1997; Ferrari *et al*., 1997). It thus seems that the formation of pores by $P2X_7$ receptors is negatively regulated in salivary glands. According to Petrou *et al*. (1997), $P2X_4$ subunits which are also expressed in acini (Tenneti *et al*., 1998; Marino *et al*., 1999) could inhibit the ability of $P2X_7$ receptors to form large pores. This is not consistent with the fact that $P2X_7$ subunits did not form hetero-oligomeric complexes (Torres *et al*., 1999). We have previously reported that ATP activates an $iPLA_2$ in acini (Kabré *et al*., 1999). In this work we show that BEL, an inhibitor of the $iPLA_2$ (Hazen *et al*., 1991) potentiated the uptake of ethidium bromide in response to ATP. BEL could also block a phosphatidate phosphohydrolase (Balsinde & Dennis, 1996). Our results suggest that the inhibition by BEL of the lipase rather than the phosphatidate hydrolase is involved in the potentiation of the response to ATP: (1) Similar concentrations of BEL (10 μM) were required to inhibit the $iPLA_2$ or to potentiate the uptake of ethidium bromide in response to ATP; (2) these two responses required a preincubation with BEL (Alzola *et al*., 1998; Kabré *et al*., 1999) and (3) propranolol, another inhibitor of the phosphatidate phosphohydrolase (Sozzani *et al*., 1992) did not potentiate but rather inhibited the response to ATP (Alzola *et al*., in preparation). It has been claimed that a preincubation with BEL induces profound modifications of the cellular phospholipids (Balsinde *et al*., 1995). In agreement with this hypothesis, we have shown that a preincubation with BEL nearly fully blocked the labeling of ductal cells with [^{3}H]-arachidonic acid (Alzola *et al*., 1998). It can thus be speculated that in response to an agonist, the $P2X_7$ receptors form a non-specific cation channel and activates an $iPLA_2$. This enzyme, in turn, modifies the lipid composition of the membrane preventing the movements of $P2X_7$ subunits and the formation of large pores. An alternative would be that in response to the hydrolysis of phospholipids by the $iPLA_2$, some inhibitor of the $P2X_7$ pore-forming activity would be generated.

In conclusion, the $P2X_7$ receptors present in acinar submandibular cells can induce the formation of large pores permeant to ethidium bromide. The formation of these pores is increased when the cells were incubated in the presence of an inhibitor of the $iPLA_2$, an enzyme activated by purinergic agonists. It can thus be speculated that the $iPLA_2$ could have a protective effect against the cytolytic properties of the $P2X_7$ receptor.

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