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ORIGINAL ARTICLE

Pharmacological evidence for the stimulation of NADPH oxidase by $P2X_7$ receptors in mouse submandibular glands

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Abstract ATP in the 100 μM-1 mM concentration range provoked a calcium-independent increase of the oxidation of dichlorodihydrofluorescein (DCFH) to dichlorofluorescein (DCF) by mouse submandibular cells. 3′-*O*-(4-benzoyl) benzoyl adenosine 5′-triphosphate (BzATP), a $P2X_7$ agonist, but not a muscarinic or an adrenergic agonist, reproduced the effect of ATP. The inhibition of phospholipase C by U73122 or the potentiation of $P2X_4$ receptor activation with ivermectin did not modify the response to ATP. ATP did not increase the oxidation of DCFH in cells isolated from submandibular glands of $P2X_7$ knockout mice or in cells pretreated with a $P2X_7$ antagonist. The inhibition of protein kinase C or of mitogen-activated protein kinase (MAP kinase) or of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase blocked the oxidation of DCFH without affecting the increase of the intracellular concentration of calcium or the uptake of ethidium bromide in response to extracellular ATP. From these results it is concluded that the activation of the $P2X_7$ receptors from submandibular glands triggers an intracellular signalling cascade involving protein kinase C and MAP kinase leading to the stimulation of NADPH oxidase and the subsequent generation of reactive oxygen species.

Keywords MAP kinase · NADPH oxidase · Protein kinase C · Salivary glands · Saliva

Abbreviations

ROS	reactive oxygen species
DCFH	2′,7′-dichlorodihydrofluorescein
DCF	2′,7′-dichlorofluorescein
MAP kinase	mitogen-activated protein kinase
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
DPI	diphenyleneiodonium chloride
BzATP	3′-*O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate
oATP	adenosine 5′-triphosphate 2′,3′-dialdehyde
KN-62	(1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine)
FCCP	carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone

Introduction

The production of reactive oxygen is a major response to invading micro-organisms [1]. Phagocytic cells express a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (phox) which translocates to the mem-

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brane when it is activated [2]. The enzymatic complex generates superoxide, the precursor of reactive oxygen species (ROS). These ROS contribute to the protection of the interior milieu and to the killing of invading pathogens: a deficit of phox activity provokes the chronic granulomatous disease, an inherited disease characterized by an increased sensitivity to microbial infections [3]. Among phagocytic cells, macrophages are a major source of ROS. They produce ROS not only after exposure to a pathogen but also in response to extracellular ATP [4, 5]. Non-phagocytic cells can also produce ROS [6]. Geiszt et al. [7] suggested that the release of ROS by exocrine glands might have a protective role and prevent the colonization of the lumen of these glands by bacteria. Salivary glands are at the ostium of the digestive tract. The production of saliva is essential for the maintenance of the integrity of the oral mucosa. Xerostomia is frequently associated with oral cancers and with recurrent infections of the oral cavity [8]. This is best explained by the composition of the saliva. This exocrine secretion contains not only salts and digestive enzymes (like kallikrein or amylase and lipase) but also IgA, cystatins, histatins, peroxidase, lactoferrin or lysozyme, proteins which have fungicidal, bactericidal or viricidal properties [9]. The production of ROS by these glands might also be a mechanism of defence against infections but has never been extensively studied. We and others have demonstrated that the ductal and acinar cells from these glands express various types of purinergic receptors [10, 11]. The purpose of our work was to test for the possible production of ROS by submandibular salivary glands in response to purinergic agonists. Our results show that the $P2X_7$ receptor is coupled to the production of ROS by a mechanism involving protein kinase C and mitogen-activated protein (MAP) kinase and the activation of NADPH oxidase.

Materials and methods

The experiments were carried out on C57Bl/6J $P2X_7R^{+/+}$ mice (wild-type, WT) (Charles River Laboratories, Worcester, MA, USA) and $P2X_7R^{-/-}$ (knockout, KO mice) kindly supplied by Pfizer Inc. (Groton, CT, USA). Breeding of $P2X_7$ KO mice males with females was used to maintain the colony of receptor-deficient animals. Mice used in the experiments were between 20 and 25 g (12–16 weeks of age). The expression of $P2X_7$ receptors in WT mice and its absence in KO mice were tested by Western blot as previously described [12]. The animals were fed ad libitum and had free access to water. The care and use of the animals involved in this study was approved by the Belgian Ministry of Agriculture in agreement with European regulations.

LY294002, PD98059 and bisindolylmaleimide (BIM) were purchased from Calbiochem (VWR, Leuven, Belgium). The 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH DA), fura-2/AM and ethidium bromide were from Invitrogen (Groningen, Netherlands). Allopurinol, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), apocynin, diphenyleneiodonium chloride (DPI), *N*-methylarginine, *N*-acetylcysteine, thiourea, thapsigargin, ionomycin, chelerythrine, 1-[6-[[17-β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), 1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione (U73343), adenosine 5′-triphosphate 2′,3′-dialdehyde (oATP), ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), (+) and (-) isoproterenol, phorbol 12 myristate 13 acetate (PMA) ivermectin and l-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) were purchased from Sigma (St. Louis, MO. USA). The bovine serum albumin (BSA, fraction V) was from Roche Diagnostics (Mannheim, Germany).

Preparation of the cellular suspension

The mice were killed with CO_2. The submandibular glands were immediately dissected and finely minced. The minced tissue was digested in the presence of 0.4–0.5 U/ml of collagenase P for 20 min at 37°C under constant shaking in 10 ml HEPES-buffered saline (HBS) medium containing (mM): 24.5 HEPES (pH 7.4), 96 NaCl, 6 KCl, 1 $MgCl_2$, 2.5 NaH_2PO_4, 11.5 glucose, 5 sodium pyruvate, 5 sodium glutamate, 5 sodium fumarate, 1% (v/v) glutamine-free amino acids mixture and 0.125% (w/v) BSA. Ten minutes after the beginning of the digestion, the cells were aspirated five times with 10-ml glass pipettes and further incubated for 10 min. At the end of the digestion the crude suspension was mechanically dispersed by gentle pipetting, filtered and washed in 0.9% NaCl solution.

Assay of the intracellular production of ROS

The production of ROS was estimated with DCFH which becomes fluorescent after oxidation to 2′,7′-dichlorofluorescein (DCF) by hydrogen peroxide, peroxyl radical or peroxynitrite anion [13]. One-millilitre aliquots of the cellular suspension from submandibular glands were preincubated in the dark, at 25°C for at least 1 h in the presence of 1 mM calcium and 1% (w/v) BSA and 6 μM DCFH DA, a cell-permeant analog of DCFH. After this incubation, 0.5 ml of the cellular suspension were washed once with NaCl 0.9% and the cellular pellet was resuspended in 2 ml HBS medium without BSA or magnesium but with 1 mM calcium. The assay was

performed at 25°C under constant stirring (SLM-Aminco Bowman, Urbana, IL, USA). The samples were excited at 485 nm and the light emitted at 525 nm was measured every second.

Assay of the intracellular concentration of calcium ($[Ca^{2+}]_i$)

One-millilitre aliquots of the cellular suspension were incubated in 2 ml HBS medium in the presence of 0.25 mM $CaCl_2$ and 0.5% BSA. The suspension was incubated for 45 min at 25°C in the presence of 2 μM fura-2/AM. At the end of the incubation, the cells were washed once with 10 ml isotonic NaCl. The pellet was resuspended in 4 ml fresh HBS medium, in the absence of amino acids, $MgCl_2$ or BSA but in the presence of 1 mM $CaCl_2$. The calcium assay was performed at 25°C under constant stirring on 2 ml of the cellular suspension. The excitation wavelength was switched every second from 340 to 380 nm and the light emitted at 510 nm was recorded. At the end of the assay, the traces were calibrated with the successive addition of 100 μM digitonin and 40 mM EGTA (pH 8.5 with tris). The autofluorescence measured after quenching the fluorescence of the fura-2 by the addition of 100 mM $MnCl_2$ was subtracted from all the data before calculation of the ratios. The calcium concentration was estimated by the ratio method [14].

Measurement of the permeabilization of the plasma membrane

The permeability of the plasma membrane was measured with ethidium bromide which does not normally permeate through the plasma membrane. Once inside the cells, the salt which is non-fluorescent intercalates in double-stranded nucleic acids and becomes fluorescent. The cells from two submandibular glands were resuspended in 4 ml HBS medium without calcium or magnesium. One-millilitre aliquots were used for each measurement. Ethidium bromide was added at a final concentration of 20 μM. The cells were allowed to equilibrate for 5 min before the beginning of the measurement which was performed at 37°C under constant stirring. The samples were excited at 360 nm and the light emitted at 580 nm was measured every second. At the end of the measurement, the maximum uptake was estimated by adding 100 μM digitonin and the results were calculated taking this value as reference.

Statistical analysis

The results were compared using the Mann-Whitney test ($n \leq 6$) on the Student's t-test ($n > 6$).

Results

Purinergic receptor activation by ATP increases the production of ROS

As shown in Fig. 1, cells from submandibular glands preincubated with DCFH DA emitted light at 525 nm after excitation at 485 nm. Under basal conditions, the fluorescence slightly increased with time. This increase was linear for at least 10 min (Fig. 1). The addition of 1 mM ATP to the cuvette provoked a rapid increase of the slope of the curve: the rate of the increase of the fluorescence during the 120 s following the addition of 1 mM ATP was 2.6±0.2-fold higher than the rate of the increase of fluorescence 60 s before the addition of ATP. Considering that the increase of the fluorescence was secondary to the oxidation of DCFH to DCF, extracellular ATP probably increased the production of ROS. In agreement with this hypothesis, 20 mM *N*-acetylcysteine, which is a scavenger of ROS, inhibited the increase of the fluorescence of DCF under basal conditions from $992 \pm 64 \times 10^{-6}$ arbitrary fluorescence units (AFU) ($n=43$) to $633 \pm 50 \times 10^{-6}$ AFU ($n=15$) in the presence of *N*-acetylcysteine ($P=0.043$ when comparing the means using a non-paired *t*-test). *N*-acetylcysteine fully abolished the response to ATP when the nucleotide was added to the medium 7 min after the start of the measurement (Fig. 2, upper panel). *N*-Acetylcysteine partially inhibited the fast but not the late increase of the $[Ca^{2+}]_i$ in response to ATP

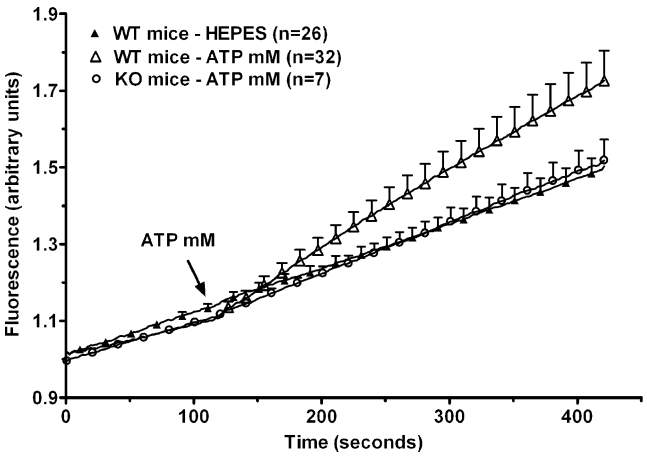

Fig. 1 Effect of ATP on the oxidation of DCFH. Cells from submandibular glands of WT (*triangles*) or KO (*circles*) mice were loaded with DCFH. After washing, the cells were resuspended in 2 ml HBS medium in the absence of magnesium, in the presence of 1 mM calcium and transferred in the cuvette of a spectrofluorimeter. The fluorescence emitted at 525 nm after excitation at 485 nm was measured every second. Two minutes after the start of the measurement, some cells were exposed to 1 mM ATP (*open symbols*). Results are expressed as arbitrary units of fluorescence and are the means ± SEM of *n* experiments

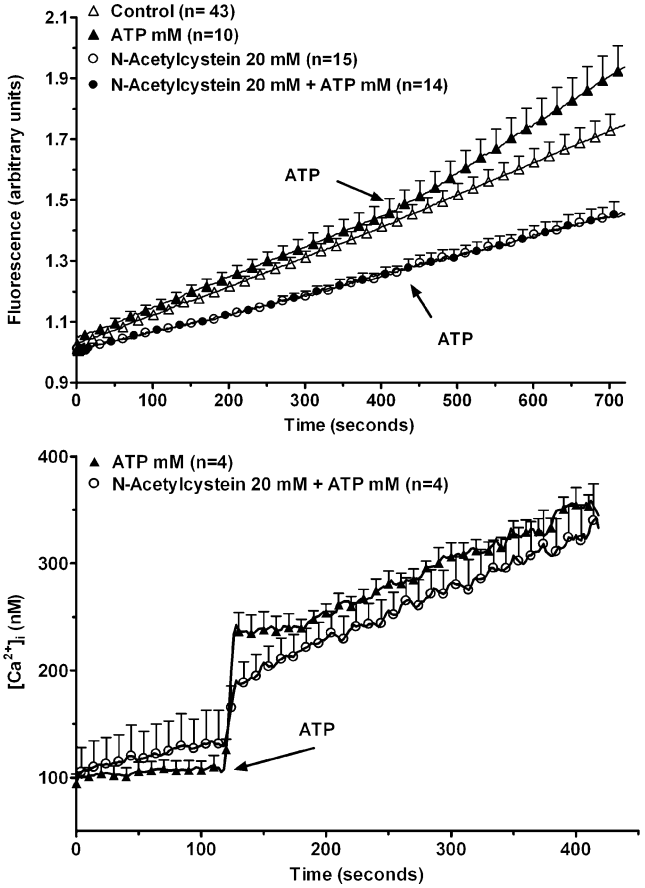

Fig. 2 Effect of N-acetylcysteine on the response of mouse submandibular glands to extracellular ATP. *Upper panel*: cells preloaded with DCFH were resuspended in HBS medium in the absence of magnesium and in the presence of 1 mM calcium and in the absence (*triangles*) or in the presence (*circles*) of 20 mM *N*-acetylcysteine. Seven minutes after the start of the measurement the cells were stimulated with 1 mM ATP. Results are expressed as arbitrary units of fluorescence and are the means ± SEM of *n* experiments. *Lower panel*: cells were preloaded with fura-2 and resuspended in HBS medium in the absence of magnesium, in the presence of 1 mM calcium and in the absence (*triangles*) or in the presence (*circles*) of 20 mM *N*-acetylcysteine. Two minutes after the start of the measurement the cells were stimulated with 1 mM ATP. At the end of the measurement, the traces were calibrated with the successive addition of digitonin, EGTA and manganese. Results are the means ± SEM of *n* experiments

(Fig. 2, lower panel). The increased oxidation of DCFH was specific for purinergic agonists (Fig. 3). Carbachol, an agonist of muscarinic receptors, had no effect on the rate of DCFH oxidation (n=5); (-) isoproterenol, a β-adrenergic agonist, decreased the slope of the curve to 44±2% (n=6). This inhibition was reproduced in two experiments with (+) isoproterenol, the isomer inactive on adrenergic receptors (34 and 46% inhibition in two experiments) but not by forskolin, a general activator of adenylate cyclase (100± 4%, n=8). These results suggested that isoproterenol might be a scavenger for ROS.

Characterization of the purinergic receptors involved in the production of ROS

Salivary glands express both metabotropic ($P2Y_1$ and $P2Y_2$) and ionotropic ($P2X_4$ and $P2X_7$) purinergic receptors [10, 11]. To determine whether the receptor involved in the production of ROS was a P2Y and/or a P2X receptor, the cells were exposed to U73122, an inhibitor of phospholipase C (PLC) or its analog, U73343, which has no effect on PLC [15]. As shown in Fig. 3, these two aminosteroids did not affect the response to ATP. In a next step we studied the effect of ivermectin on the response to ATP. In submandibular glands, this anti-parasite drug strongly potentiates the response to $P2X_4$ activation [12]. Ivermectin at a 100 μM concentration did not modify the production of ROS in response to ATP (269±11%, n=4), suggesting that $P2X_7$ rather than $P2X_4$ receptors triggered this response. As shown in Fig. 4, concentrations of ATP higher than 100 μM were required to observe a significant increase of the rate of DCFH oxidation upon ATP stimulation. The $P2X_7$ receptor differs from the six other P2X receptors not only by its structure (it has a long intracellular C-terminal domain which is absent in the other P2X receptors) but also by the fact that it has a very low sensitivity to ATP^{4-} [16]. The response to ATP significantly dropped from 249±15%, n=29, to 130±6%, n=4, P<0.01) following the addition of magnesium to the medium which forms $ATPMg^{2-}$ and decreases the concentration of ATP^{4-} (Fig. 4). KN-62 and

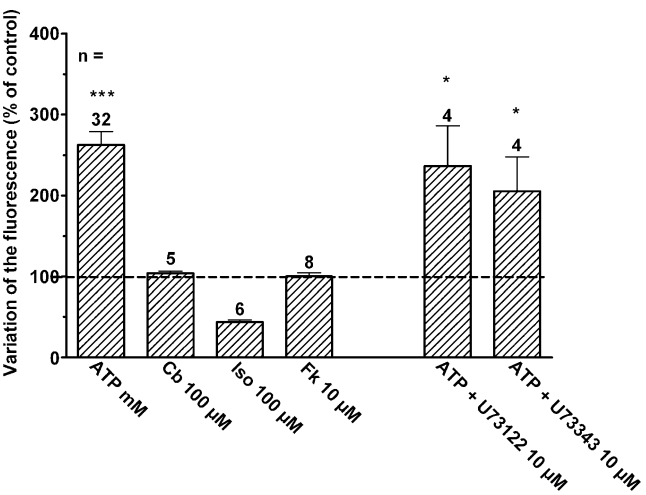

Fig. 3 Effect of various agonists on the oxidation of DCFH by mouse submandibular cells. Cells were treated as described in the legend to Fig. 1. They were resuspended in HBS medium under control conditions or in the presence of 10 μM U73122 or U73343. Two minutes after the start of the measurement, the cells were exposed to the mentioned agonists. The results are expressed as the ratio of the slope of the curve after the addition of 1 mM ATP and the slope of the curve before the addition of 1 mM ATP. Results are the means ± SEM of *n* experiments. *P<0.05; ***P<0.005

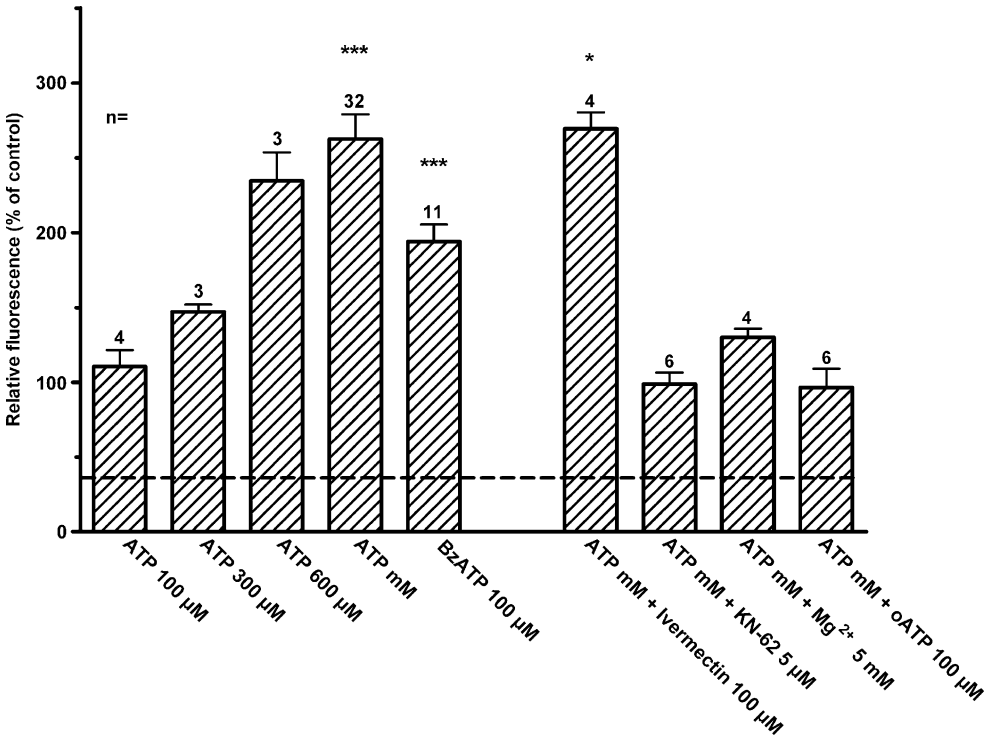

Fig. 4 Role of $P2X_7$ receptors on the oxidation of DCFH. Cells preloaded with DCFH were resuspended in HBS with 5 mM magnesium or in HBS medium in the absence of magnesium, under control conditions or in the presence of either 100 µM ivermectin, 5 µM KN-62 or 100 µM oATP. Two minutes after the start of the measurement the cells were exposed to the mentioned concentrations of ATP or to 100 µM BzATP. Results are the means ± SEM of *n* experiments. $^{**}P<0.01$; $^{***}P<0.005$

oxidized ATP, two inhibitors of $P2X_7$ receptors, also blocked the production of ROS in response to extracellular ATP (down to 99±8% for KN-62 and 96±12% for oATP, $n=6$). The $P2X_7$ receptor, unlike the other P2X receptors, is much less sensitive to ATP than to its analog, 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate (BzATP). As shown in Fig. 4, 100 µM BzATP increased the production of ROS to a level nearly similar to the level reached by 1 mM ATP. All these results strongly suggested that $P2X_7$ receptors were involved in the production of ROS. This hypothesis was fully confirmed with $P2X_7$ KO mice. As originally shown by the group of Gabel [17] with macrophages, submandibular cells from these mice did not express $P2X_7$ receptors (data not shown). The exposure of these cells to ATP did not increase their capacity to oxidize DCFH (Fig. 1).

Mechanisms coupling the activation of $P2X_7$ receptors to the production of ROS

We have previously reported that the activation of $P2X_7$ receptors in submandibular glands provokes a massive uptake of extracellular calcium responsible for a sustained increase of the intracellular concentration of the ion [18]. The incubation of the submandibular cells in the absence of calcium but in the presence of 500 µM EGTA, a calcium chelator, had no effect on the production of ROS in response to ATP ($P=0.6688$), suggesting that ATP did not increase the production of ROS after increasing the $[Ca^{2+}]_i$ (Fig. 5, upper panel). When cells were incubated in the presence of extracellular calcium, ionomycin, which is a calcium ionophore, and thapsigargin, which blocks Ca^{2+}-ATPases in endoplasmic reticulum, did not increase the rate of DCFH oxidation (92±15%, $n=3$, and 79±8%, $n=5$, when compared to the rate before the addition of ionomycin or thapsigargin, respectively). Under the same experimental conditions, both ionomycin and thapsigargin increased the $[Ca^{2+}]_i$ (data not shown). These results confirmed that calcium ions play no role in the production of ROS in response to ATP. There is a general consensus that the production of ROS by monocytes/macrophages in response to ATP is regulated by kinases [5]. Therefore, inhibitors of various kinases were next tested (Fig. 5, lower panel). The inhibition of the phosphatidylinositol-3 kinase by LY294002 [19] had no effect on the response to ATP (from 182±11%, $n=20$, in the presence of DMSO to 199±17%, $n=10$, in the presence of 100 µM LY294002). Bisindolylmaleimide, an inhibitor of protein kinase C dose-dependently blocked the response to ATP in the 100 nM-10 µM concentration range. Chelerythrine, another inhibitor of protein kinase C, also inhibited the response to 1 mM ATP. PD98059, a potent, cell-permeant and selective inhibitor of p42/44 MAP kinase [20], blocked the response to ATP (down to 133±11%, $n=10$, $P<0.005$ in the presence of 100 µM PD98059). Chelerythrine and PD98059 which inhibited the production of ROS in response to ATP did not affect the increase of the $[Ca^{2+}]_i$ or the uptake of ethidium bromide provoked by the nucleotide (data not shown)

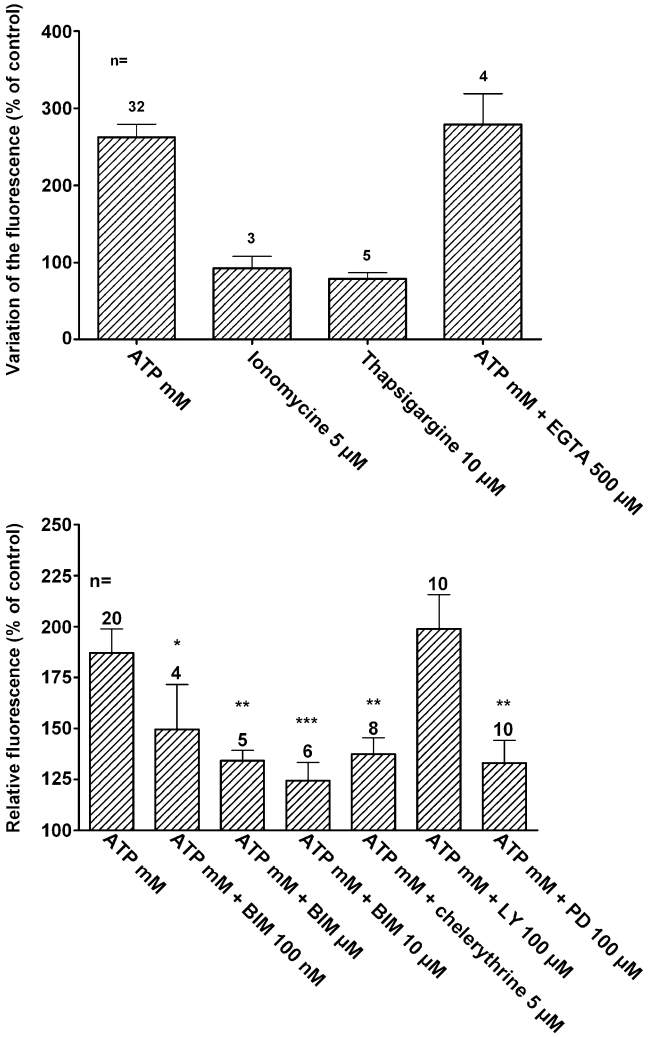

Fig. 5 Intracellular pathway mediating the response to $P2X_7$ receptors. *Upper panel*: cells preloaded with DCFH were resuspended in HBS without calcium but in the presence of 500 µM EGTA or in HBS with 1 mM calcium. Two minutes after the start of the measurement cells were exposed to 1 mM ATP or 5 µM ionomycin or 10 µM thapsigargin. Results are the means + SEM of *n* experiments. *Lower panel*: cells preloaded with DCFH were resuspended in HBS medium containing 1 mM calcium. The cells were exposed for 10 min at 25°C to control conditions (DMSO) or to various concentrations of bisindolylmaleimide (BIM) or to 5 µM chelerythrine or 100 µM LY294002 or to 100 µM PD98059. Two minutes after the start of the measurement cells were exposed to 1 mM ATP. Results are the means ± SEM of *n* experiments. **P*<0.05; ***P*<0.01; ****P*<0.005

Identification of the source of ROS produced by ATP stimulation

Several mechanisms (electron transport system, enzymatic activities) could contribute to the production of ROS. Several inhibitors were tested for their possible effect on the oxidation of DCFH in response to ATP. The preincubation of the submandibular glands with 1 µM oligomycin, an inhibitor of the proton channel of the mitochondrial ATPase or 1 µM FCCP, a protonophore uncoupling the mitochondria, or with 1 mM allopurinol, an inhibitor of xanthine oxidase, or with 1 mM *N*-methylarginine, an inhibitor of nitric oxide synthase, had no effect on the response to ATP (Fig. 6). Apocynin (100 µM), an inhibitor of the translocation of the p47 subunit of NADPH oxidase from the cytosol to the plasma membrane [21], fully blocked the response to ATP (from 186±15%, n=11, in the presence of ATP alone to 101±8%, n=4, in the presence of ATP plus apocynin). One millimolar AEBSF, an inhibitor of the assembly of the various subunits of NADPH oxidase [22], or 500 µM DPI, an inhibitor of NADPH oxidase and other flavoproteins [23], also inhibited the stimulation of ROS production by ATP (143±4%, n=8, for AEBSF and 112±13%, n=4, for DPI, $P<0.05$ for both inhibitors when compared to ATP plus DMSO). AEBSF and DPI had no effect on the increase of the $[Ca^{2+}]_i$ or the uptake of ethidium bromide in response to ATP mM. Apocynin interfered with the assay of the $[Ca^{2+}]_i$ with the fura-2 method. It had no effect on the uptake of ethidium bromide provoked by ATP (data not shown).

Discussion

We have shown in this work that the rate of oxidation of DCFH was increased when cells from submandibular glands of control mice were exposed to ATP. It is generally agreed that the oxidation of DCFH is secondary to the production of ROS [24, 25]. However, this method has its limitations: DCFH itself can generate ROS under aerobic conditions [26, 27]. We evaluated the contribution of ROS in the oxidation of DCFH by incubating the cells with a ROS scavenger, *N*-acetylcysteine. This drug significantly inhibited the basal increase of the fluorescence of DCFH by about 30%. It also abolished the increase of fluorescence provoked by ATP and inhibited the initial increase of the $[Ca^{2+}]_i$ following the exposure of the submandibular cells to ATP. The increase of the $[Ca^{2+}]_i$ seen after 1-min exposure to the agonist was not affected by *N*-acetylcysteine. We have recently reported that the initial component of the response to ATP mainly involved $P2X_4$ receptors; $P2X_7$ receptors mostly contribute to the later phase of the response [12]. The results obtained with *N*-acetylcysteine suggest that this drug probably inhibited the interaction between ATP and the $P2X_4$ receptor, not the $P2X_7$ receptor. The various P2X receptors have ten cysteine residues forming five disulfide bridges which are essential for the receptor function or its trafficking to the plasma membrane [28]. Our results suggest that *N*-acetylcysteine might reduce some of the disulfide bridges of the $P2X_4$ receptor and affect the interaction between this receptor and its agonist.

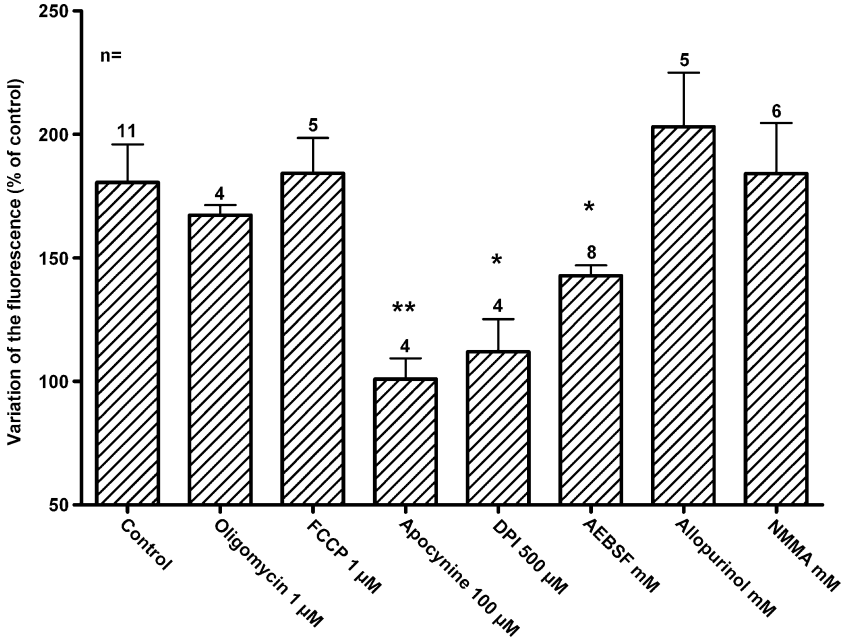

Fig. 6 Mechanism involved in the oxidation of DCFH by ATP. Cells preloaded with DCFH were resuspended in HBS medium containing 1 mM calcium. The cells were exposed for 10 min at 25°C to control conditions (DMSO) or to the antagonists mentioned in the figure. Two minutes after the start of the measurement cells were exposed to 1 mM ATP. Results are the means ± SEM of *n* experiments. **P*<0.05; ***P*<0.01

Isoproterenol, which is a β-adrenergic agonist, inhibited rather than increased the basal fluorescence of DCF. This is consistent with the results of Barroso et al. [29] who showed that the activity of superoxide dismutase was increased in submandibular glands from isoproterenol-treated animals. It should however be noted that the response to isoproterenol was probably not secondary to the activation of the adrenergic receptor and the stimulation of adenylate cyclase since it was reproduced with the inactive isomer of isoproterenol and since forskolin, which is a general activator of adenylate cyclase, had no effect on the fluorescence of DCF. These results suggest that isoproterenol itself might have antioxidative properties thanks to its phenol group [30].

The production of ROS in response to extracellular ATP required high concentrations (higher than 100 μM) of the nucleotide. The response to ATP was blocked by extracellular magnesium, KN-62 or oATP; it was reproduced by BzATP and was not sensitive to U73122, an inhibitor of PLC, or to ivermectin, an enhancer of the activation of $P2X_4$ receptors by ATP. Importantly, it was not reproduced in cells from $P2X_7$ receptors KO mice. All these results suggest that the production of ROS in response to ATP is mediated by $P2X_7$ receptors. These receptors are expressed in exocrine glands and especially in submandibular glands [12]. They form a non-selective cation channel and their occupancy leads to a massive uptake of extracellular calcium and the increase of the $[Ca^{2+}]_i$. In a secondary step, these receptors form a pore which is permeant to ethidium bromide [10]. The response to ATP was not affected by the removal of extracellular calcium, suggesting that the production of ROS was not secondary to the increase of the $[Ca^{2+}]_i$. This is consistent with the results obtained with carbachol. This muscarinic agonist transiently increased the $[Ca^{2+}]_i$ without modifying the production of ROS. Thapsigargin and ionomycin, which both provoked a sustained increase of the $[Ca^{2+}]_i$, had no effect on the rate of DCFH oxidation, confirming that calcium ions are not involved in the stimulation of ROS production. The cascade leading from receptor occupancy to ROS production probably involved protein kinase C and p42/44 MAP kinase since inhibitors of these two enzyme activities decreased the production of ROS in response to ATP without affecting the non-selective cation channel or the pore coupled to the $P2X_7$ receptors. This is in agreement with the results of Bradford and Soltoff who nicely demonstrated the involvement of protein kinase C and p42/44 MAP kinase activation in the cellular signalling pathway triggered by $P2X_7$ activation in parotid glands [31]. It should however be mentioned that the activation of protein kinase C is necessary but probably not sufficient to stimulate the production of ROS since in our hands the incubation of the cells with a phorbol ester (PMA) which activates protein kinase C had no effect on fluorescence of DCFH (data not shown). The combination of PMA with ionomycin was also without effect on the production of ROS. These results are consistent with the results observed with carbachol: the muscarinic agonist which increases the activity of protein kinase C [31] has no effect on the production of ROS (vide supra). It should be recalled that, contrary to carbachol, the responses triggered by agonists of the $P2X_7$ receptors are numerous and pleiotropic. These agonists not only increase the phosphorylation of proteins but also provoke major changes in the ionic composition of

the cytosol (sodium, potassium, calcium, protons), in the metabolism of phospholipids or in the permeability of the plasma membrane [32].

After the exclusion of several sources of ROS (mitochondria, xanthine oxidase, NO synthase) in the response to ATP, the possibility remained that NADPH oxidase or one of its isoforms might contribute to the response to extracellular ATP. Indeed it has been shown that exocrine glands among which submandibular glands express one of the homologs of the $gp91^{phox}$ subunit of NADPH oxidase, the "dual" oxidase 2 (Duox2) [33]. Duox2 not only contains the NADPH oxidase domain but also a domain that is homologous to heme-containing peroxidases such as myeloperoxidase and lactoperoxidase, hence its name [34]. When compared to the $gp91^{phox}$, Duox2 has a large N-terminal extension comprising two EF-hand motifs, which explains its regulation by calcium ions [35]. Considering that calcium had no effect on the production of ROS by ATP, Duox2 was probably not involved in this response. Three inhibitors of NADPH oxidase, DPI or apocynin or AEBSF, inhibited the production of ROS but not the increase of the $[Ca^{2+}]_i$ or the uptake of ethidium bromide in response to extracellular ATP. From these results we conclude that NADPH oxidase is the enzyme responsible for the production of ROS by submandibular glands exposed to ATP.

$P2X_7$ receptors are thus coupled to the production of ROS not only in phagocytic cells [4, 5, 36] but also in non-phagocytic cells (this report). In both cell types the NADPH oxidase is the source of these ROS, but the signalling pathway activating this enzyme might differ among these cells. Parvathenani et al. [36] reported that in microglial cells the response to ATP was also calcium dependent; the MAP kinase mediating the response to ATP was not attenuated by PD98059, an inhibitor of p42/44 MAP kinase, but by SB203580, an inhibitor of p38 MAP kinase; it was more sensitive to an inhibitor of PI3 kinase. In phagocytic cells ROS participate in host defence by killing invading microorganisms and by triggering the release of cytokines [37]. In salivary glands, the production of ROS in response to purinergic agonists (this report) and the secretion of peroxidase in response to autonomic agonists (muscarinic, adrenergic, tachykininergic or VIPergic) [38] by the cells might contribute to the production of hydrogen peroxide which could kill pathogens present in the oral cavity [7].

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