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Contribution of two ionotropic purinergic receptors to ATP responses in submandibular gland ductal cells

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Abstract

The effect of extracellular ATP on salivary gland function was compared in wild-type (WT) and $P2X_7$ knockout (KO) mice. The increase in the intracellular concentration of calcium ($[Ca^{2+}]_i$) in response to carbachol was similar in submandibular ductal cells of WT and KO mice. ATP and its analog, benzoyl-ATP, induced a sustained increase in the $[Ca^{2+}]_i$ in WT animals. In KO mice, ATP slightly and transiently increased the $[Ca^{2+}]_i$ and benzoyl-ATP had no effect. The response to ATP of WT but not KO mice was blocked by KN-62, Coomassie blue and magnesium. The small response of ATP observed in KO mice was completely blocked in the absence of extracellular calcium, unchanged by U73122 and potentiated by ivermectin indicating the probable involvement of a $P2X_4$ receptor. A RT-PCR and a Western blot confirmed the presence of these receptors in ducts of both WT and KO mice. ATP increased the permeability of the cells to ethidium bromide and stimulated a phospholipase A_2 activity in WT but not KO mice. Mice submandibular gland cells secreted IL-1β but this secretion was not modified by ATP and was similar in both groups of animals. The volume of saliva provoked by pilocarpine and the concentration of proteins, sodium and chloride in this saliva was similar in both groups of animals. The concentration of potassium was higher in KO mice.

We can conclude that the major purinergic receptors expressed in mice submandibular ductal cells are $P2X_7$ receptors but that $P2X_4$ receptors are also involved in some ATP effects.

Keywords: $P2X_7$ receptors; KO mice; Purinergic; IL-1β; Saliva; Exocrine glands

1. Introduction

Purinergic receptors have been classified in 2 major groups [1]. The metabotropic receptors (P2Y) have seven transmembrane domains and are coupled to phospholipase C or adenylate cyclase [2]. The ionotropic receptors (P2X) have only two transmembrane domains. The assembly of these receptors as homo- or hetero-polymers forms a non-specific cation channel. Seven P2X receptors have been cloned and their distribution is ubiquitous. Among these receptors, the $P2X_7$ receptor is unique sharing only 40% homology with other P2X receptors [3]. The major structural differences of the $P2X_7$ receptor are located at the intracellular C-terminus which is much longer than in other receptors [4]. This cytoplasmic tail contains several domains [5] which favor the interaction of this receptor with intracellular proteins [6]. The

Abbreviations: AEBSF; 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; BSA; bovine serum albumin; Bz-ATP; 2′-3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; $[Ca^{2+}]_i$; intracellular concentration of calcium; EDTA; ethylene diamine tetraacetic acid; EGTA; ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HBS; HEPES-buffered saline; IL-1β; interleukin-1β; KN-62; 1-(*N*,*O*-bis-[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenyl-piperazine; LPS; lipopolysaccharide; $P2X_7R^{+/+}$; $P2X_7$ receptor wild-type mice: WT mice; $P2X_7R^{-/-}$; $P2X_7$ receptor knockout mice: KO mice; PBS-T; phosphate buffered saline Tween-20; PMSF; phenylmethylsulfonyl fluoride; PPI-PLC; polyphosphoinositide-phospholipase C.

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C-terminus of $P2X_7$ receptor is also responsible for the formation of a pore in the plasma membrane which allows the passage of charged molecules under 900 Da [7]. The $P2X_7$ receptors are expressed by various blood cells [8]. This receptor is also detected in epithelial cells [9]. Acinar and ductal cells from salivary glands express not only this receptor [10] but also $P2Y_1$, $P2Y_2$ and $P2X_4$ receptors [11]. This diversity of purinergic receptors expressed by salivary glands and the lack of pharmacological tool truly specific for $P2X_7$ receptors have considerably complicated the study of the role of these receptors in the physiology of salivary glands. In spite of these obstacles, in vitro data have established that these receptors might regulate ion fluxes [12] and protein secretion [13]. Considering the low affinity of ATP for $P2X_7$ receptors, their physiological role has also been questioned. The availability of mice which do not express $P2X_7$ receptors (KO mice) has contributed to a better understanding of the role of these receptors in major physiological processes. These KO mice have a phenotype which is apparently normal [14]. The role, in vivo, of $P2X_7$ receptors in IL-1β release from peritoneal macrophages [15] or the role of these receptors in the inflammatory response [16] or in the cell death of microglia or macrophages [17] were demonstrated using these KO mice. These mice were also used as a model to confirm the importance of $P2X_7$ receptors in bone resorption and remodeling [14].

The purpose of this work was to use this animal model to investigate the contribution of $P2X_7$ receptors in the response of salivary ductal cells to extracellular ATP. A recent work of Yao et al. [18] demonstrated the presence of IL-1β in mice submandibular gland and the role of kallikrein mK13 in its maturation. We thus decided to test the effect of ATP and $P2X_7$ receptors on IL-1β secretion by mice submandibular glands. Finally, we studied the role of these receptors in the composition of saliva. Our results show that $P2X_7$ receptors are the major purinergic receptors present in mice submandibular ductal cells and that $P2X_4$ receptors also mediate part of ATP action. They also show that $P2X_7$ receptors are involved in the regulation of the ionic composition of saliva but probably not in the secretion of IL-1β.

2. Materials and methods

2.1. Preparation of the ductal and acinar suspension

The mice were sacrificed with CO_2 asphyxiation. The submandibular glands crude suspension was prepared as previously described [10,19]. Ductal cells were separated from acinar cells by centrifugation at 4000 *g* at 4 °C for 10 min through an isotonic Percoll solution (40%). The ducts, which remained on the top of the Percoll, and the acini, which migrated to the bottom of the Percoll, were recovered, washed and resuspended in 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) bovine serum albumin (BSA). They were kept at 4 °C until use.

2.2. Uptake of ethidium bromide

Ductal cells from 3 mice were resuspended in 3 ml HBS medium. For each assay, a 1-ml aliquot was washed and resuspended in 2 ml HBS medium without magnesium chloride, amino acids or BSA. The permeabilization of the plasma membrane was measured with the fluorescent dye ethidium bromide as previously described [19].

2.3. Intracellular calcium concentration ($[Ca^{2+}]_i$)

After their isolation, submandibular ductal cells from 3 mice were resuspended in 2 ml fresh HBS medium. One ml of this suspension was incubated in 2 ml HBS medium in the presence of 0.25 mM calcium chloride, 0.5% BSA and 2 μM fura-2/AM for 45 min at room temperature. The calcium assay was performed as previously described [20] on 1 ml of the cellular suspension. The calcium concentration was estimated by the ratio method as described by Grynkiewicz et al. [21].

2.4. Phospholipase A_2 activity

The activity of phospholipase A_2 was followed as previously described [22] by measuring the release of radioactivity from ductal cells prelabelled with [^{3}H] oleic acid.

2.5. Membrane preparation

Ducts and acini from 4 mice were resuspended in 1 ml ice-cold TEEI buffer (20 mM Tris–HCl pH 8, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 2.5 μg/ml aprotinin, 2.5 μg/ml pepstatin A, 2.5 μg/ml leupeptin) and passed through a 29G needle. The cells were centrifuged at 1000 ×*g* for 10 min at 4 °C. The pellet was reextracted with 1 ml ice-cold TEEI buffer and the supernatants were combined. This operation was repeated 4 times. The four supernatants were centrifuged at 100 000 ×*g* for 30 min at 4 °C. The membrane pellet was resuspended in 200 μl (ducts) or 400 μl (acini) ice-cold TEEI containing 0.3% Triton-X-100 for 30 min at 4 °C to extract the membrane proteins and then passed through a 29G needle. The lysate was centrifuged at 10 000 ×*g* for 10 min at 4 °C to remove aggregates.

2.6. Preparation of submandibular extracts for IL-1β immunoblot

A crude suspension of submandibular glands of 3 mice was prepared as described in a previous paragraph. The cells were incubated for 2 h at 37 °C in HBS medium without amino acids, magnesium chloride and albumin but containing 1 μg/ml LPS. The cells were then centrifuged at 10 000 ×*g* for 10 min. The supernatants were treated with a protease inhibitor cocktail diluted 400× (containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 1.5 mM pepstatin A, 4 mM bestatin and 1.4 mM E-64) and stored at −20 °C.

2.7. Western blot analysis of $P2X_4$ receptors and IL-1β protein expression

Twenty μl 4x NuPage LDS sample buffer were added to 4 μl 2-mercaptoethanol and to 60 μl membrane lysate ($P2X_4$ receptors) or 60 μl supernatant (IL-1β). The samples were then heated at 80 °C for 10 min ($P2X_4$ receptors) or at 40 °C for 1 h (IL-1β). The samples were centrifuged at 10 000 ×*g* for 10 min. Twenty μg membrane proteins ($P2X_4$ receptors) or 45 μg proteins in the supernatant (IL-1β) were separated by electrophoresis at 200 V for 50 min on NuPage® Bis-Tris 4-12% gels ($P2X_4$ receptors) or Bis-Tris 12% gels (IL-1β). Proteins were then electrophoretically transferred to a 0.2 μm nitrocellulose membrane ($P2X_4$ receptors) or to a 0.45 μm PVDF membrane (IL-1β) at 30 V for 60 min. The membranes were blocked for 2 h at room temperature in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl and 0.1% Tween 20) containing 5% of non-fat dried milk powder. After 2 washes with PBS-T, the membranes were exposed overnight at 4 °C to 2 μg/ml anti-$P2X_4$ receptor antibody or to 0.2 μg/ml anti-IL-1β antibody in PBS-T with 2.5% non-fat dried milk. After 5 washes with PBS-T, the membranes were incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:10 000 dilution, $P2X_4$ receptors) or with peroxidase-conjugated donkey anti-goat IgG (1:6000 dilution, IL-1β) for 1 h in PBS-T with 2.5% non-fat dried milk. After another run of 5 washes, the immunoreactive proteins were visualized on X-ray films (BioMax MR films, Kodak) with a chemiluminescent horseradish peroxidase substrate (ECL Plus).