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The effect of anions on the human P2X7 receptor

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ABSTRACT

P2X7 receptors (P2X7Rs) are nonselective cation channels that are opened by the binding of extracellular ATP and are involved in the modulation of epithelial secretion, inflammation and nociception. Here, we investigated the effect of extracellular anions on channel gating and permeation of human P2X7Rs (hP2X7Rs) expressed in *Xenopus laevis* oocytes. Two-microelectrode voltage-clamp recordings showed that ATP-induced hP2X7R-mediated currents increased when extracellular chloride was substituted by the organic anions glutamate or aspartate and decreased when chloride was replaced by the inorganic anions nitrate, sulfate or iodide. ATP concentration–response comparisons revealed that substitution of chloride by glutamate decreased agonist efficacy, while substitution by iodide increased agonist efficacy at high ATP concentrations. Meanwhile, the ATP potency remained unchanged. Activation of the hP2X7R at low ATP concentrations via the high-affinity ATP effector site was not affected by the replacement of chloride by glutamate or iodide. To analyze the anion effect on the hP2X7R at the single-molecule level, we performed single-channel current measurements using the patch-clamp technique in the outside-out configuration. Chloride substitution did not affect the single-channel conductance, but the probability that the P2X7R channel was open increased when chloride was replaced by glutamate and decreased when chloride was replaced by iodide. This effect was due to an influence of the anions on the mean closed times of the hP2X7R channel. We conclude that hP2X7R channels are not anion-permeable in physiological Na^+-based media and that external anions allosterically affect ion channel opening in the fully ATP^4-liganded P2X7R through an extracellular anion binding site.

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1. Introduction

P2X7 receptors (P2X7Rs), which are expressed in secretory epithelial cells [1] and in the cells of the immune and inflammatory systems [2], are nonspecific cation channels. P2X7Rs are opened by extracellular ATP, which is secreted into the extracellular space under pathological conditions such as cell necrosis, hypoxia or platelet aggregation [3]. P2X7R activation is involved in several pathophysiological processes, including the release of the inflammation-promoting interleukin 1ß (IL-1β), the killing of intracellular bacteria, the formation of multinuclear giant cells, renal fibrosis, and neuropathic and inflammatory pain [2]. In the dorsal horn of the spinal cord, ATP released from strongly activated or damaged afferent nociceptive neurons may activate microglial cells via P2X7Rs [4]. Because of its involvement in these pathological processes, P2X7R has become a promising pharmacological target [5] for the treatment of inflammation and pain.

P2X7R activation is modulated by extracellular organic signaling molecules, which may upregulate (e.g., lipopolysaccharide and interferon-γ [6], IL-1β [7], TNF-α [8]) or downregulate (e.g., IL-4 and Il-10 [8]) P2X7R-dependent signaling. However, inorganic ions can also influence P2X7R-mediated cellular events [9]. Divalent cations such as Ca^{2+}, Zn^{2+}, Cu^{2+}, and Mg^{2+}[10–12], as well as protons [13,14], inhibit P2X7R activation. Replacement of extracellular Cl^- ions by I^- reduces the P2X7R-mediated ion current [15], the uptake of the cationic dye Yo-Pro-1 [14], and the processing and secretion of IL-1β [16]. The substitution of extracellular Cl^- with the organic anions glutamate (Glu^-), aspartate (Asp^-) or gluconate (Glc^-) exhibits inconsistent effects. It increases P2X7R-dependent ion currents in the rat neuroblastoma–mouse glioma cell line [15] and in the FRTL rat thyroid cell line [17], but it reduces P2X7R-mediated currents in murine parotid acinar and duct cells [18]. hP2X7R-dependent Yo-Pro-1 [14,19] and rat P2X7R (rP2X7R)-mediated ethidium$^+$ uptake [20] is increased by Cl^- replacement with Glu^- or Glc^-, respectively. In hP2X7R-expressing HEK cells, the replacement of extracellular Cl^- by Glu^- or Asp^- increases the potency of the agonist BzATP, while substitution

Abbreviations: ATP^{4-}, free form of ATP, not bound to cations; hP2X7R, human purinergic P2X7 receptor

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by iodide decreases potency [14]. Likewise, the replacement of Cl^- by Glc^- enhances the P2X7R-dependent secretion of IL-1β in primary mouse bone marrow-derived macrophages and in THP-1 human monocytes/macrophages [16]. In contrast, the substitution of extracellular Cl^- by gluconate$^-$ suppresses P2X7R activation-induced cell shrinkage in murine splenic T lymphocytes [21] and in chicken DT40 lymphocytes expressing rP2X7R, in which rP2X7R-mediated LDH release is also blocked [20].

The effect of extracellular Cl^- substitution on P2X7R-mediated cell function may be complex. Apart from the direct effects of anions on the P2X7R, indirect effects may include the secondary activation of other ion channels that modulate ATP-induced ion fluxes and membrane potential changes. Accordingly, the exchange of an extracellular anion species may affect ion fluxes through anion channels that are co-expressed with P2X7Rs in immune and epithelial cells [22]. In HEK 293 cells expressing the mouse P2X7R, ATP-induced anion currents have been reported [23]. The substitution of Cl^- by large organic anions such as Glu^-, which normally permeate anion channels less readily than the smaller Cl^- [24], will lead to increased Cl^- efflux accompanied by cell membrane depolarization. The resulting diminished inwardly directed driving force for cations would decrease all P2X7R-mediated effects that are due to Na^+ or Ca^{2+} influx. In contrast, a more pronounced depolarization would increase the K^+ efflux, which is believed to be involved in IL-1β processing [2]. Furthermore, prolonged application of P2X7R agonists leads to an increase in cell membrane conductance, which seems to be mediated by dilatation of the P2X7R ion channel pore or P2X7R-dependent activation of other ion-conducting membrane proteins [9]. To address these issues, we examined the effects of anions on the function of the hP2X7R, which was heterologously expressed in *Xenopus laevis* oocytes. By using the voltage-clamp technique, membrane potential-dependent effects could be excluded. The use of oocytes in Ca^{2+} and Mg^{2+}-free extracellular solutions also simplified the experimental conditions and prevented the activation of Ca^{2+}-dependent ion currents. To gain insight into the effect of extracellular anions on the hP2X7R at the molecular level and to avoid overlay of the measured ATP-dependent current by ion conductances other than those mediated by the P2X7R, we also performed single-channel current measurements. The anion species-induced alterations of the macroscopic current conductance were reflected at the single-channel level by changes in the probability of hP2X7 channel-opening events.

2. Materials and methods

2.1. Expression of the hP2X7R and its mutants in X. laevis oocytes

Linearized templates of oocyte expression plasmids encoding the wild-type hP2X7R subunit (accession no. Y09561 [25]) and its $hP2X7R^{S339Y}$ mutant [13] were transcribed into capped cRNA as previously described [26]. Collagenase-defolliculated stage V or VI oocytes [13] were injected with 2 ng of the wild-type or mutant hP2X7R cRNA and maintained at 19 °C in Barth's solution (100 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) until they were used 1–3 days later.

The maintenance of and operation on frogs were approved by the local animal welfare committee (ref no. 53a-42502/2-173) and are in compliance with EC Directive 86/609/EEC for animal experiments.

2.2. Two-electrode voltage-clamp (TEVC) recordings

Microelectrodes filled with 3 M KCl with resistances of 0.8–1.2 MΩ were impaled into oocytes superfused with frog Ringer's solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4). Currents were recorded at room temperature (~22 °C) using an oocyte clamp OC-725C amplifier (Warner Instruments, Hamden, USA), filtered at 100 Hz and sampled at 85 Hz, essentially as previously described [13]. Switching between the different bathing solutions was achieved within less than 1 s by a set of computer-controlled magnetic valves using a modified U-tube technique [26].

Subsequent measurements of the hP2X7R-dependent currents were performed in bathing solutions consisting of 100 mM NaX, 5 mM HEPES, and 0.1 mM EGTA, pH 7.4, where X denotes the extracellular anion that was used. In the case of the divalent anion SO_4^{2-}, the Na_2SO_4 concentration was reduced to 70 mM for osmotic reasons. Ca^{2+}-free solutions were supplemented with 0.1 mM flufenamic acid to block the conductance evoked by external divalent cation removal. The hP2X7R-mediated inward currents were elicited by switching for 6 s to a bathing solution that also contained free (i.e., non-complex) ATP (ATP^{4-}) at the concentrations indicated in the text and figures. The interval between agonist applications was usually 4 min.

Ramp currents were measured during 300-ms-long voltage ramps applied every 1 s between −60 and +40 mV. Between the ramps, the holding potential was maintained at −40 mV. The ATP-induced ramp currents were calculated as the difference between the ramp currents before and during the ATP application.

2.3. Single-channel recordings

Single-channel currents were recorded using the patch-clamp technique in the outside-out configuration using an Axopatch 1D patch clamp amplifier as previously described (Axon Instruments, Foster City, Ca., USA) [27]. Currents were filtered at 1 kHz and sampled at 5 kHz. Patch pipettes were pulled from borosilicate glass, coated with Sylgard (Dow Corning Corp., Midland, MI, USA) and filled with a solution consisting of 90 mM aspartic acid, 10 mM CsCl, 10 mM EGTA, 10 mM BAPTA, 10 mM HEPES and 0.5 mM $MgCl_2$, pH 7.2 (adjusted with CsOH). The resistances of the patch pipettes were 10–15 MΩ when measured in frog Ringer's solution.

The bathing solutions consisted of 100 mM NaX, 0.5 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 (adjusted with NaOH/HCl), where X denotes the salt anion. ATP-evoked currents were elicited by manually placing the tip of the patch pipette into the outflow stream of a U-tube containing the bathing solution plus 0.1 mM ATP^{4-}. Steady-state single-channel currents were recorded 1 s after ATP application.

Ramp currents were measured during voltage ramps of 1 s between −120 and +60 mV, which were applied every 2 s. Between the ramps, the holding potential was maintained at −120 mV. Ramp currents without channel openings were subtracted to remove capacitive and leak currents from the values.

The reference electrodes were connected to the bath by 3 M KCl–agar bridges. The remaining diffusion potentials were measured according to a previously published method [28]. In Glu- or Asp-based extracellular solutions, this potential was ~−10 mV and was corrected accordingly. The diffusion potential for bathing solutions containing other anions was negligible.

2.4. Data analysis

The data were stored and analyzed on a personal computer. For ion current recording and analysis, a software system developed in our department was used (Superpatch 2000, SP-Analyzer by T. Böhm). For the construction and analysis of single-channel current histograms, the computer program ASCD was used (generously provided by G. Droogmans, Catholic University Leuven, Belgium) [27]. The SigmaPlot program (SPSS, Chicago, USA) was used for non-linear approximations and graphical representations of the data. The data are reported as the mean ± SEM. The statistical data

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