Regulation of the P2X7 Receptor Permeability to Large Molecules by Extracellular Cl\textsuperscript{−} and Na\textsuperscript{+}\textsuperscript{*}

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Upon continuous stimulation, the pore of the monovalent cation-selective P2X7 receptor (P2X7R) expands to accommodate large molecules such as N-methyl-D-glucamine (NMDG\textsuperscript{+}). How the change in P2X7R permeability is regulated is not known. Here we report that extracellular Cl\textsuperscript{−} (Cl\textsubscript{o}) regulates the outward current, whereas extracellular Na\textsuperscript{+} (Na\textsubscript{o}) regulates the inward current of large molecules by P2X7Rs. The P2X7R-mediated current was measured in parotid acinar and duct cells of wild type and P2X7R\textsuperscript{−/−} mice and in HEK293 cells expressing the human or mouse P2X7R isoforms. In symmetrical NaCl, triethylammonium chloride, and NMDG\textsuperscript{+} chloride solutions, the P2X7R current followed a linear current/voltage relationship. In symmetrical NaCl, removal of Cl\textsubscript{o} reduced the inward Na\textsuperscript{+} current by \( \sim 35\% \) and the outward Na\textsuperscript{+} current by only \( 10\% \). By contrast, in the absence of Na\textsuperscript{o} and the presence of Na\textsubscript{i}, or NMDG\textsuperscript{+}, the removal of Cl\textsubscript{o} reduced the inward Na\textsuperscript{+} or NMDG\textsuperscript{+} currents by \( 35\% \) but the outward NMDG\textsuperscript{+} current by \( > 95\% \). The effect of Cl\textsubscript{o} was half-maximal at \( \sim 60 \text{ mm} \). Reducing Cl\textsubscript{o} from 150 to 10 mm did not reproduce the effects of Cl\textsubscript{o} removal. All currents were eliminated in P2X7R\textsuperscript{−/−} cells and reproduced by expressing the P2X7Rs in HEK cells. These findings suggest that Cl\textsubscript{o} primarily regulates the outward P2X7R current of large molecules. When cells dialyzed with NMDG\textsuperscript{+} were stimulated in the presence of Na\textsubscript{o} and subsequent removal of Na\textsubscript{o} resulted in a strongly outward rectifying NMDG\textsuperscript{+} current, indicating maintained high selectivity for Na\textsuperscript{+} over NMDG\textsuperscript{+}. During continuous incubation in Na\textsuperscript{+}-free medium, the permeability of the P2X7Rs to NMDG\textsuperscript{+} gradually increased. On the other hand, when the cells were incubated in symmetrical NMDG\textsuperscript{+} and only then stimulated with ATP, the NMDG\textsuperscript{+} current by P2X7Rs followed a linear current/voltage relationship and did not change with time. These findings suggest that the P2X7R has a “Na\textsuperscript{+} memory” and that Na\textsuperscript{+} regulates the inward permeability of P2X7Rs to large molecules. The novel regulation of P2X7R outward and inward permeability to large molecules by Cl\textsubscript{o} and Na\textsubscript{o}, respectively, may have an important protective function, particularly in secretory epithelial cells.

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The ionotropic P2X7 receptors (P2X7Rs)\textsuperscript{1} function as non-selective cation channels that are activated by high concentrations of ATP and by the relatively specific agonist 3’-O-(4-benzoyl)benzoyl ATP (1). P2X7Rs participate in several cell functions. Activation of P2X7Rs in hematopoietic cells leads to cytokine release, cell permeabilization, and apoptosis (2). In bone P2X7Rs have been implicated in the generation of osteoclasts (3), and in epithelial cells they may modulate secretory and growth function (4, 5). In addition, after prolonged activation with ATP, the P2X7Rs become permeable to large molecules with molecular weights as high as 1000 (1). Association between increased permeability to large molecules and induction of apoptosis was reported in several cell types (1).

Epithelial cells of secretory glands secrete ATP to both the basal and luminal spaces (6, 7) that can activate the P2 receptors in the respective membranes. Furthermore, neutrophils and epithelial cells (8), including salivary glands (9), synthesize and secrete the antimicrobial cathelicidins. The cathelicidin-derived antimicrobial peptide LL37 appears to be an activator of the P2X7Rs (10). Hence, activation of the P2X7Rs can play an important role in the inflammatory response observed in epithelial autoimmune disorders such as Sjogren’s syndrome (11). Understanding the function and regulation of P2X7Rs in epithelial cells is of intrinsic interest and may also impact the understanding of tissue function in normal and pathologic states.

Like many epithelial cells (1, 7), parotid acinar and duct cells express P2X7Rs (5, 12, 13). Recently, we immunolocalized the P2X7Rs in the luminal membrane of parotid acinar and duct cells (13). Similar localization was reported in the pancreatic duct (14) and uterine epithelial cells (15), whereas P2X7Rs were found at the basal pole of outer hair cells (16). The P2X7Rs showed remarkable cell-specific behavior in native cells (13). For example, the first activation of P2X7R current by ATP proceeds very slowly in parotid acinar cells, but duct cells from the same gland respond very rapidly to the first application of ATP. The P2X7Rs appear to interact differentially with the cytoskeleton in parotid acinar and duct cells (13). However, the P2X7Rs in both cell types show similar pharmacology, such as similar activation by ATP, sensitivity to external divalent ions, permeability to Na\textsuperscript{+}, and inhibition by Brilliant Blue G, Cu\textsuperscript{2+}, and pyridoxal phosphate-6-azophenyl-2’,4’-disulfonyl acid tetrasodium (13). The cell-specific behavior of the receptors further highlights the need to understand the function and regulation of the P2X7Rs in native tissues.

A central function of salivary glands is Ca\textsuperscript{2+}-stimulated fluid secretion fueled by transcellular ion transport (17, 18). Salivary gland acinar cells secrete isotonic NaCl-rich fluid. The duct

\textsuperscript{1} The abbreviations used are: P2X7R, P2X7 receptor; Cl\textsuperscript{−}, extracellular Cl\textsuperscript{−}; Cl\textsubscript{i}, intracellular Cl\textsuperscript{−}; hP2X7, human P2X7; I/V, current/voltage; mP2X7, mouse P2X7; Na\textsubscript{o}, extracellular Na\textsuperscript{+}; Na\textsubscript{i}, intracellular Na\textsuperscript{+}; NMDG\textsuperscript{−}, N-methyl-D-glucamine; NMDGCl, NMDG chloride; TEA, tetraethyl ammonium; TEACl, TEA chloride; WT, wild type.
modifies the electrolyte composition and osmolarity of the secreted fluid by absorbing the Na\(^+\) and Cl\(^-\) and secreting K\(^+\) and HCO\(_3^\)\(^-\) (17, 18). The function of both cell types is mediated by Cl\(^-\) and K\(^+\) channels. It is well established that the channels in acinar cells are activated by the M3 receptors, which increase [Ca\(^{2+}\)]\(_i\). However, activation of P2X7R and other P2 receptors expressed in these cells also increases [Ca\(^{2+}\)]\(_i\), which can then activate the Cl\(^-\) and K\(^+\) channels (4, 14, 19, 20). Ductal secretion is controlled by the cystic fibrosis transmembrane conductance regulator (CFTR), which is activated by a \(\beta\)-adrenergic receptor-mediated increase in cAMP (17, 18). Acinar and duct cells (18, 21, 22) express additional Cl\(^-\) and K\(^+\) channels that may also be activated by P2 receptors to participate in electrolyte and fluid transport by acinar and duct cells. In this respect, it was reported recently that the stimulation of parotid acinar cells with high concentrations of ATP or 3-O-(4-benzoyl)benzoyl ATP activates an apparent Cl\(^-\) current with a pharmacology different from that of P2X7Rs and from any known Cl\(^-\) channel, leading to the conclusion that parotid acinar cells express a novel Cl\(^-\) channel that is activated by high concentrations of ATP (22).

As part of our effort to understand the role of P2X7Rs in salivary gland physiology, we re-examined the effect of ATP on cation and anion currents in parotid acinar and duct cells of WT and P2X7R\(^-/-\) cells and in HEK293 cells transfected with the hP2X7Rs and mP2X7Rs. We report that a high concentration of ATP activates only cation current by native P2X7Rs and from any known Cl\(^-\) channel, leading to the conclusion that parotid acinar cells express a novel Cl\(^-\) channel that is activated by high concentrations of ATP (22).

**EXPERIMENTAL PROCEDURES**

**Materials**—The hP2X7R clone was generously provided by Dr. George Dubiak (Case Western University, Cleveland, OH), and the mP2X7R clone was from Dr. Friedrich Haag (Institute of Immunology, University Hospital, Hamburg, Germany). Na\(^-\)-ATP and Tris-ATP were from Sigma. The following solutions were used in these experiments. The standard bath solution, solution A, contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). ATP was added as 5 mM Na\(^-\)-ATP. Na\(^-\)-free bath solution was prepared by replacing all Na\(^+\) salts with the respective gluconate salts. Solutions containing various anions were prepared by replacing all Cl\(^-\) salts with the respective gluconate salts. Solutions containing various anions were prepared by replacing all Cl\(^-\) salts with the respective anion salts. The solutions containing tetraethyl ammonium (TEA) were prepared as reported by Arreola and Melvin (22) to contain 140 mM TEACl, 0.5 mM CaCl\(_2\), 100 mM mannitol, and 20 mM Heps, pH 7.3, with toxicity of 375 mosmol. The TEA pipette solution contained 140 mM TEACl, 20 mM EGTA, and 20 mM Heps, pH 7.3, with toxicity of 335 mosmol. The standard pipette solution contained 140 mM KCl, 1 mM NaCl, 1 mM MgCl\(_2\), 10 mM EGTA, 5 mM ATP, and 10 mM HEPES (pH 7.4 with NaOH). The Na\(^-\)-containing and Na\(^-\)-free pipette and bath solutions for recording of Na\(^+\) and NMDG\(^+\) currents, respectively, were identical and contained 150 mM NaCl or 10 mM NMDGCl, 5 mM ATP, and 10 mM HEPES (pH 7.4 with NaOH or NMDGOH). Osmolarity values of all solutions were adjusted to 310 mosmol with the major salt. The P2X7R\(^-/-\) mice were generously provided by Dr. Christopher Gabel (Pfizer, Groton, Connecticut) and were maintained on standard mice chow per National Institutes of Health regulations. All animal protocols were approved by the animal use committee of University of Texas Southwestern Medical Center.

**Culture and Transfection of HEK293 Cells**—HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The cells were co-transfected with hP2X7Rs or mP2X7Rs and a plasmid coding for green fluorescent protein. Green fluorescent protein fluorescence was used to identify the transfected cells. Transient transfection was by Lipofectamine. Cells were used 36–48 h post-transfection.

**Preparation of Single Parotid Acinar and Duct Cells**—Parotid acinar and duct cells were prepared by a standard enzymatic digestion as was done previously (23, 24), with minor modifications (13). Mouse parotid glands were minced and treated with 0.025% trypsin for 7 min at 37 °C. Trypsin digestion was terminated by washing the cells with a solution containing 1.5 mg/ml soybean trypsin inhibitor. Then the tissue was digested for 20 min at 37 °C with 70 units/ml collagenase CLSPA. The cells were washed, suspended in solution A containing 1.5 mg/ml soybean trypsin inhibitor, and kept on ice until use.

**Current Recording**—Current was recorded using the whole cell mode of the patch-clamp technique at the conditions described previously (23, 24). All pipette solutions contained 10 mM EGTA except for the TEACl pipette solution, which contained 20 mM EGTA to reproduce the conditions of Arreola and Melvin (22). Pipette resistance was 3–7 megaohms, and the access resistance was ~10 megaohms. Tight seals were formed on the cell surface, and the whole cell configuration was obtained by gentle suction. Current was recorded using the Axopatch 200B patchclamp amplifier. Data were collected at 5 kHz and filtered at 1 kHz. During whole cell recording the membrane potential was held at ~60 or ~0 mV, as specified. The membrane conductance was probed by stepping the membrane potential from a holding potential of 0 mV to membrane potentials between ~80 and +80 mV at 10-mV steps for 200 msec with 500-msec intervals between steps. Current recording and analysis was performed with pClamp 6.0.3 software, and Figs. 1–7 were prepared with Origin 7.5.

**RESULTS**

**P2X7Rs Mediate the Current in Symmetrical TEACl Solutions**—Previous work reported an apparent activation of a Cl\(^-\) current by stimulation of mouse parotid acinar cells with high concentrations of ATP or with 3'-O-(4-benzoyl)benzoyl ATP. The current was attributed to Cl\(^-\), because the cells and bath solution contained TEA as the major cation and Cl\(^-\) as the only anion. It was concluded that the Cl\(^-\) current was not mediated by the P2X7Rs present in these cells based on inhibition of the ATP-activated Na\(^+\) current and not of the apparent Cl\(^-\) current by Cibacron blue 3G A (22). We re-examined the current activated by a high concentration of ATP in the same cell type and in parotid duct cells obtained from WT and P2X7R\(^-/-\) mice. Fig. 1, A and B recapitulate the findings of Arreola and Melvin (22), showing activation by 5 mM ATP of an outward current in parotid acinar cells held at ~80 mV and incubated in symmetrical TEACl solutions. Fig. 1, C and D show that similar current can be measured in parotid duct cells. Notably, Fig. 1 also shows that the current measured under these conditions was not present in P2X7R\(^-/-\) cells, providing the first evidence that
the current is mediated by P2X7Rs.

The current observed in Fig. 1 can be mediated by the P2X7Rs or by another channel that is activated by the P2X7Rs. To distinguish between these possibilities we tested whether expression of recombinant human and mouse P2X7Rs will result in current with the same characteristics. In preliminary experiments using HEK293 cells transfected with hP2X7Rs and mP2X7Rs and incubated in symmetrical NaCl solutions, we found that the kinetic of activation of the hP2X7Rs and mP2X7Rs by ATP is different and resembles the kinetics that we found in the parotid acinar and duct cells, respectively (13). Thus, the first exposure of hP2X7Rs to ATP resulted in slow current activation, but subsequent removals and additions of ATP caused rapid inhibition and activation of the current, as was found in parotid acinar cells (13). On the other hand, mP2X7R was rapidly activated upon the first application of ATP, as was found in the parotid duct cells (13). Fig. 2 shows that similar behavior is evident when the HEK293 cells expressing hP2X7Rs (panels A and B) or mP2X7Rs (panels C and D) were incubated in symmetrical TEACl solutions and stimulated with ATP.

Other observations of note in Fig. 2 are that expression of the P2X7Rs activated an outward current similar to that found in WT cells and that removal of Cl\textsuperscript{−} partially reduced the outward current (Fig. 2C), as was found in parotid acinar cells (22). Based on the findings in Figs. 1 and 2 we conclude that the outward current observed under the conditions of Figs. 1 and 2 and in the experiments (22) is carried by the P2X7Rs.

P2X7Rs Are Not Permeable to Cl\textsuperscript{−}.—P2X7Rs function as a non-selective monovalent cation channels. However, shortly after activation they became permeable to large molecules like NMDG\textsuperscript{+} and YO-PRO-1 (1). P2XR may also conduct anions, as was reported recently for the permeability of P2X5Rs to Cl\textsuperscript{−} (25). Therefore, the outward current recorded in the presence of TEACl in parotid acinar and duct cells and in HEK293 cells expressing P2X7Rs can be carried by TEA\textsuperscript{−} or Cl\textsuperscript{−}. The protocol in Fig. 3 was designed to distinguish between these possibilities. Parotid acinar and duct cells were dialyzed and incubated in the same solution containing 150 mM NaCl, held at −60 mV, and stimulated with ATP. Acinar and duct cells showed the typical slow and fast current activation, respectively, upon the first application of ATP (13). Removal and re-addition of external Na\textsuperscript{+} showed that under these conditions most of the current was carried by Na\textsuperscript{+}. The membrane potential was then switched to 0 mV, and the cells were stimulated with ATP. The lack of current at 0 mV indicated that the cells were thoroughly dialyzed with the pipette solution. Removal of Na\textsuperscript{+} generated the expected outward current that was nullified by the re-addition of Na\textsuperscript{+} to the bath. Removal of Cl\textsuperscript{−} also generated an apparent small outward current (Fig. 3, A and C). Identical results were obtained in HEK293 cells expressing hP2X7Rs or mP2X7Rs (not shown), and the sustained currents were abolished in P2X7R\textsuperscript{−} cells (Fig. 3, B and C). The initial transient currents that are resolved very well in the P2X7R\textsuperscript{−} cells are likely due to expression of P2X4Rs in these cells (26, 27), which rapidly desensitize upon continuous stimulation with ATP (1).

If P2X7Rs were permeable to Cl\textsuperscript{−}, removal of Cl\textsuperscript{−} under the conditions of Fig. 3 should have generated an inward current. Therefore, the results in Fig. 3 indicate that in the presence of 150 mM Na\textsuperscript{+} and Na\textsuperscript{+} the P2X7R has no Cl permeability. However, the unexpected outward current observed upon removal of Cl\textsuperscript{−} suggested that Cl\textsuperscript{−} may regulate P2X7R cation permeability. To further examine the effect of Cl\textsuperscript{−} on P2X7R function, we measured the Cl\textsuperscript{−} dependence and the anion selectivity of this effect. The results are represented in Fig. 4. Parotid acinar and duct cells held at −60 mV were stimulated with ATP to verify maximal activation of P2X7R current. Subsequent current recording at a membrane potential of 0 mV while changing Cl\textsuperscript{−} between 0 and 150 mM showed that maximal outward current was observed by reducing Cl\textsuperscript{−} to 61 ± 4 mM (n = 5) (Fig. 4, A and C). The selectivity of the anion-activation of P2X7Rs followed the sequence Cl\textsuperscript{−} > Br\textsuperscript{−} > SO\textsubscript{4}\textsuperscript{2−} > formate > I\textsuperscript{−} > NO\textsubscript{3}\textsuperscript{−} > glutamate (Fig. 4, B and D).
In the current at 0 mV in parotid acinar (A and B) and duct (C and D) cells. In panels A and C the cells were exposed to different external Cl⁻ concentrations, whereas in panels B and D medium Cl⁻ was replaced with the indicated anions at 150 mM, i.e. glutamate (Glu), Br⁻, I⁻, NO₃⁻, and formate (formate). Current density in parotid acinar and duct cells, respectively, similar to those described for panels A and B were used to calculate the percentage of inhibition of the inward (Na⁺) and outward Na⁺ currents and NMDG⁺ current (Na⁺). The effects of different Cl⁻ concentrations and of the anions are displayed in a 5× expanded current scale. The traces represent at least five experiments with similar results.

13%, resulting in a shift in the reversal potential from +0.6 ± 0.4 to -8.4 ± 0.7 mV (n = 9). These findings explain the apparent outward current measured upon removal of Cl⁻ at the membrane potential of 0 mV shown in Fig. 3.

The sidedness of the effect of Cl⁻ on the P2X7R Na⁺ currents (activating the inward more than the outward current) raised the questions of whether the effect was specific to Cl⁻ and whether Cl⁻ always preferentially activated the inward current. To address the first question, we measured the effect of reducing Cl⁻ from 150 to 10 mM on current density and on the effect of the removal of Cl⁻. Current density in parotid acinar cells held at -80 mV and, stimulated with 5 mM ATP, was not affected by reducing Cl⁻, averaging 52.7 ± 6.4 and 56.3 ± 5.8 pA/pF (n = 5) in the presence of 150 and 10 mM Cl⁻, respectively. Furthermore, Fig. 5, C and D show that Cl⁻ did not affect the extent of the sidedness of activation of P2X7Rs by Cl⁻. In addition, reducing Cl⁻ to 10 mM did not shift the reversal potential in symmetrical 150 mM Na⁺ solutions.

Next, we examined the effect of Cl⁻ on inward Na⁺ and NMDG⁺ currents and the outward NMDG⁺ current. Fig. 6, A (acinar) and B (duct) show that removal of Cl⁻ inhibited the inward Na⁺ current in the presence (Fig. 5) and absence (Fig. 6) of Na⁺, to a similar extent. By contrast, although Cl⁻ only minimally affected the outward Na⁺ current (Fig. 5), it markedly activated the outward NMDG⁺ current. Thus, as summarized in Fig. 6D, removal of Cl⁻ reduced the inward Na⁺ current by ~35% and the outward NMDG⁺ current by >95% in both parotid acinar and duct cells. In Fig. 6C we compared the effect of Cl⁻ on the inward and outward NMDG⁺ currents. Again, it is evident that removal of Cl⁻ reduced the outward current much more than the inward NMDG⁺ current (Fig. 6D).

Hence, the effect of Cl⁻ was independent of the presence or absence of Na⁺. Important additional information is given in Fig. 6E, showing that removal of Cl⁻, which almost abolished the outward current, only minimally changed the reversal potential. Under the conditions of Fig. 6, A and B, a Cl⁻ current by P2X7Rs as a result of the removal of Cl⁻ would have resulted in an increased inward current and a shift in the reversal potential to a more positive value.

Na⁺ Memory of P2X7Rs—As we were characterizing the Na⁺ current we noticed that when parotid cells or HEK cells expressing P2X7Rs were dialyzed and bathed in Na⁺-free solutions, the P2X7Rs current followed a nearly linear I/V relationship between -80 and +80 mV, with a reversal potential close to 0 mV (Figs. 1, 2, and 6C). Conversely, when Na⁺ was re-
moved from the media of stimulated cells dialyzed with a Na+ free solution, the inward current was nearly abolished, the current showed strong outward rectification, and the reversal potential shifted by ~60 mV (compare filled squares and open circles in Fig. 6, A and B). A possible explanation of this behavior is a time-dependent change in P2X7R channel permeability (1) or an effect of Na+ on the activity of the P2X7Rs. The results in Fig. 7 support the latter possibility and suggest that P2X7Rs have a “Na+ memory”. For these experiments we used duct cells, because the P2X7Rs in these cells are rapidly activated upon the first stimulation with ATP (Ref. 13 and Figs. 3 and 4). The cells were used to measure the time-dependent change in the properties of the P2X7R current of cells dialyzed and incubated in Na+-free media. Fig. 7A shows that when the cells were incubated in Na+-free medium prior to stimulation with ATP, the I/V relationship of the current stimulated by ATP was nearly linear and did not change with time. By contrast, Fig. 7B shows that when cells incubated in Na+-containing medium were first stimulated with ATP and only then deprived of Na+ (10 μM), the I/V relationship measured immediately or even 2 min after the removal of Na+ showed strong outward rectification, whereas that measured 10 min after the start of the incubation in Na+-free media was linear between ~80 and +80 mV. The time course of the change in inward and outward NMDG+ currents is shown in Fig. 7C. Notably, switching the cells from Na+–containing to Na+-free media and continuous incubation in Na+-free medium increased only the inward NMDG+ current (Fig. 7C).

**DISCUSSION**

In the present work we describe novel regulatory mechanisms of P2X7Rs function that may have important physiological roles, particularly in secretory epithelia. This work began with an examination of whether stimulation of parotid acinar cells with a high concentration of ATP activates a novel anion/Cl– channel. A previous work reported activation by ATP of an outward current in cells dialyzed and incubated with symmetrical TEACl solutions that was reduced by the removal of Cl– and was independent of P2X7Rs (22). We confirmed these findings in parotid acinar cells (Fig. 1A) and extended them to parotid duct cells (Fig. 1B). However, our results show that this activity is not mediated by a novel Cl– channel but rather reflects novel regulation of the P2X7R function by extracellular ions. That the current was mediated by P2X7Rs is concluded from elimination of the current in P2X7R−/− parotid acinar and duct cells (Fig. 1) and, conversely, from replication of the current by expression of either the hP2X7Rs or the mP2X7Rs in HEK293 cells (Fig. 2).

The following findings suggest that the current was carried by the large cations (TEA+ or NMDG+) rather than Cl–. (a) Removal of Cl–, under symmetrical Na+ concentrations and at a membrane potential of 0 mV generated a small outward current (Fig. 3). A Cl– current should have generated an inward current. (b) Removal of Cl– reduced both the inward and outward currents. Cl– permeability by P2X7Rs should have reduced the outward current but increased the inward current. (c) The selectivity to anions in activating the current did not follow that of an anion channel; in particular, formate and SO42− were more effective than I− and NO3− in supporting the current (Fig. 4). (d) Reducing [Cl–]o from 150 to 10 mM did not reduce the outward current and did not shift the reversal potential (Fig. 5C). (e) Removal of Cl– from the media of cells dialyzed and incubated in symmetrical Na+–free solutions had minimal effect on the reversal potential (Fig. 6C). (f) Removal of Na+ from the media of cells stimulated with ATP resulted in a selective development of inward current (Fig. 7B). These six properties (a–f) are not compatible with a Cl– current and are all compatible with a cation current. In this respect, it is important to note that it is well established that upon continuous stimulation the P2X7R pore undergoes time-dependent expansion to increase the permeability to large cations like NMDG+ (1, 28, 29). Therefore, we conclude that, in the absence of Na+ and/or Na+o, the P2X7R has substantial permeability to TEA+ or NMDG+ and, importantly, the activity of the P2X7R is regulated by Cl– and Na+.

Our findings indicate that only Cl– regulates the P2X7R activity, because reducing [Cl–]o to 10 mM did not reproduce the effect of reducing Cl–. Activation of P2X7R current by Cl– showed clear sidedness; Cl– regulated the outward current of large molecules but not that of Na+, and regulation by Cl– is independent of Na+o. Hence, removal of Cl– reduced the inward Na+ and NMDG+ currents by the same extent, had minimal effect on the outward Na+ current, but markedly reduced the NMDG+ outward current; i.e. the outward permeability of P2X7Rs to large molecules. Together, these findings suggest that the P2X7R or a protein that interacts with the P2X7R has an extracellular Cl– binding site that controls the conformation of the P2X7R to change its regulation by voltage and to convert its conductance of large molecules from a strongly inward rectifying current in the absence of Cl–o to a linear I/V in the presence of Cl–. It is significant that reduction of Cl–o from 150 mM to the more physiological Cl– concentration in the systemic fluid of 100 mM was sufficient to induce 30% of the Cl– effect (Fig. 4, A and B), suggesting that the regulation of P2X7R activity by Cl–o occurs at physiological Cl– concentrations. An effect of Cl– on P2X7R conformation is also suggested by the effect of Cl–o on the apparent affinity of the P2X7R to ATP. Reducing Cl–o from 150 to 0 mM increased the potency of ATP in stimulating the P2X7R by an order of magnitude (30).

An interesting mode of regulation of the P2X7R is its Na+ memory (Figs. 6 and 7). That is, when the P2X7R is stimulated...

Fig. 7. Na+ memory of P2X7Rs. Parotid duct cells dialyzed with 0 Na+ solution were incubated in 0 Na+ (NMDGCl−) medium before stimulation with 5 mM ATP (A) or incubated in medium containing 150 mM NaCl, stimulated with ATP, and then switched to Na+−free medium containing ATP (B). The I/V relationships were measured before cell stimulation (B) and at different times after stimulation. Panels A and B show the I/V curves after 2 (C) and 10 min (D) of stimulation with ATP. Panel C summarizes the inward and outward currents recorded at ~80 and +80 mV, respectively, at the indicated times of three experiments. Note that current recorded with protocol (A) did not change with time, whereas the current recorded with protocol (B) show selective time-dependent activation of the inward current to convert the outward rectifying current to a linear I/V. Con, control.
in the presence of Na\(^+\), it behaves as a strongly outward rectifying channel with respect to large molecules like NMDG\(^+\). This state relaxes upon incubation of the P2X7R in the absence of Na\(^+\) for ~10 min. The state of linear current of large molecules is very stable; once it was attained, incubating the cells with 150 mM Na\(^+\) for as long as 20 min in the resting or ATP-stimulated state failed to recover the outward rectifying state (not shown). The simplest interpretation of these findings is that the P2X7R has an extracellular Na\(^+\) site that is different from the conductive pathway. This site can bind Na\(^+\) at the resting state. The Na\(^+\) becomes occluded upon stimulation of the P2X7R by ATP to control the selectivity of the P2X7R and prevent the influx of large molecules into the cell. When the P2X7R is stimulated in the absence of Na\(^+\), the external Na\(^+\) binding site may bind NMDG\(^+\) or it may remain empty while becoming unavailable to Na\(^+\). Dissociation of NMDG\(^+\) or reopening of the site is a slow process. Irrespective of the mechanism, regulation of P2X7R permeability to large molecules by Na\(^+\) can have an important physiological role.

The two novel regulatory mechanisms of the P2X7R conductance described here complement each other; Cl\(^-\) regulates the outward permeability and Na\(^+\) the inward permeability of P2X7R to large molecules. These modes of regulation may function to limit fluxes of large molecules during stimulation of the P2X7R. Stimulation of P2X7R in macrophages (31), red blood cells (32), and most likely other cells that express P2X7Rs results in the rapid loss of the intracellular K\(^+\) and in the cell shrinkage that is critical for the induction of apoptosis (31). Under these circumstances, maintaining Cl\(^-\), at or below 100 mM will reduce the efflux of vital large ions while maintaining the K\(^+\) efflux. At the same time, Na\(^+\) will prevent entry of large molecules present in the extracellular milieu. Such a potential mechanism was indeed reported very recently in DT40 cells stably expressing P2X7Rs (33). Stimulation of the P2X7Rs depolarized the cells, inducing pore formation, cell shrinkage, and massive release of lactate dehydrogenase to induce apoptosis. Remarkably, cell shrinkage and the release of lactate dehydrogenase were abolished by the removal of Cl\(^-\) (33).

Regulation of P2X7Rs activity by Cl\(^-\) and Na\(^+\) may play an even more profound role in secretory epithelia such as the salivary gland and the pancreas. The P2X7R is believed to be expressed in the luminal membrane of these cells (13, 14). Acinar cells secrete isotonic fluid containing 150 mM Na\(^+\) and 100 mM Cl\(^-\). The ducts absorb the Cl\(^-\) and in salivary glands the Na\(^+\) to reduce the Cl\(^-\) of the secreted fluid to 20 mM and to secrete moderately hypotonic fluid (17, 18, 34). Sweat ducts also absorb the Na\(^+\) and Cl\(^-\) to secrete hypotonic fluid (35). In these cells, the P2X7R can be activated either by secreted ATP (6, 7) or by cathelicidin-derived antimicrobial peptides (8–10) to increase cation permeability and depolarize the luminal membrane. Depolarization of the membrane will aid in controlling Cl\(^-\) absorption and HCO\(_3\) secretion (36). In salivary glands the P2X7R can also participate in Na\(^+\) absorption and K\(^+\) secretion. At the same time, the reduction of luminal Cl\(^-\) and the presence of Na\(^+\) or K\(^+\) in the secreted fluid will effectively seal the duct by inhibiting the efflux and the influx, respectively, of large molecules to protect the duct and possibly the acinar cells against damage caused by expansion of the P2X7R pore. These regulatory mechanisms may become compromised in disease states such as cystic fibrosis and Sjogren’s syndrome in which Cl\(^-\) absorption and electrolyte secretion are impaired. This may lead to persistent efflux of vital large molecules to further damage the glands.

In summary, the present work has revealed the regulation of P2X7R permeability to large molecules by Cl\(^-\) and Na\(^+\), which may guard against cell damage and apoptosis during the physiological stimulation of the P2X7R. The mechanism by which Na\(^+\) and Cl\(^-\) regulate P2X7R permeability to large molecules and the full physiological significance of these forms of regulation await further studies.

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Regulation of the P2X7 Receptor Permeability to Large Molecules by Extracellular Cl− and Na+
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