Modulation of human cardiovascular outward rectifying chloride channel by intra- and extracellular ATP

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Liu GX, Vepa S, Artman M, Coetzee WA. Modulation of human cardiovascular outward rectifying chloride channel by intra- and extracellular ATP. Am J Physiol Heart Circ Physiol 293: H3471–H3479, 2007. First published October 12, 2007; doi:10.1152/ajpheart.00357.2007.—The macroscopic volume-regulated anion current (VRAC) is regulated by both intracellular and extracellular ATP, which has important implications in signaling and regulation of cellular excitability. The outwardly rectifying Cl− channel (ORCC) is a major contributor to the VRAC. This study investigated the effects of intracellular and extracellular ATP on the ORCCs expressed in the human cardiovascular system. With inside-out single-channel patch-clamp techniques, ORCCs were recorded from myocytes isolated from human atrium and septal ventricle and from primary cells originating from human coronary artery endothelium and human coronary artery smooth muscle. ORCCs from all of these tissues had similar biophysical properties, i.e., they were outwardly rectifying in symmetrical Cl− solutions, exhibited a slope conductance of ~90–100 pS at positive potentials and ~22 pS at negative potentials, and had a high open probability that was independent of voltage or time. The presence of ATP at the cytosolic face of the membrane increased the number of patches that contained functional ORCC but had no effect on gating. In contrast, “extracellular” ATP (in pipette solution) had no effect on the proportion of patches in which ORCC was detected but strongly reduced the open probability by increasing the closed dwell time. The potency order for nucleotides to affect gating was ATP > ADP > AMP, which suggests that a negatively charged phosphate group is involved in ORCC block. Our findings are consistent with a role of ORCC in the human cardiovascular (atrium, ventricle, and coronary arteries). Regulation of ORCC by extracellular ATP suggests that this channel may have an important role in maintaining electrical activity and membrane potential under conditions in which extracellular ATP levels are elevated, such as with ATP release from nerve endings or during pathophysiological conditions.

human heart; atrial myocytes; ventricular myocytes; endothelial cells; smooth muscle cells

CHLORIDE CHANNELS in the cardiovascular system play an important role in maintaining the resting potential and by participating in repolarization of the action potential (15, 17, 27). There are several types of macroscopic Cl− currents identified by whole cell patch-clamp recordings, which are distinguished from each other in terms of their regulation. These include Cl− currents that are activated on receptor stimulation, volume changes, changes in membrane potential, and alterations in intracellular Ca2+ (17). A subset of Cl− channels exist that have been identified at the single-channel level with an outwardly rectifying Cl− current (ORCC) in symmetrical Cl− solution. These channels are widely distributed and have been found in many different tissues including human neurocytes (2), airway epithelium (26), lymphocytes (13), and leukemia cells (1) and in rabbit and human atrial myocytes (5, 9, 10). Since volume-regulated anion current (VRAC) and ORCC share many similarities in biophysical properties, ORCC was thought to be, at least in part, responsible for VRAC in atrial myocytes (5, 9), which may be an important potential therapeutic target for the treatment of heart disease (3). Previous studies have demonstrated that whole cell VRACs of intestine epithelial cells are regulated by both intracellular and extracellular ATP (16, 18, 22, 30). Here we examined ORCC of human cardiovascular tissue in detail at the single-channel level and characterized its modulation by intra- and extracellular ATP. We found ORCC to be present in both human atrial and ventricular myocytes, as well as in primary endothelial and smooth muscle cells originating from the human coronary artery. We found ORCC activity to be upregulated by intracellular ATP but inhibited by extracellular nucleotides.

MATERIALS AND METHODS

Human atrial and ventricular myocyte isolation. The investigation conforms to the principles outlined in the Declaration of Helsinki. Human heart tissue was obtained from a total of seven patients undergoing open-heart surgery suffering from valvular or congenital heart disease. The age range was between 3 mo and 63 yr. Tissue was obtained with Institutional Review Board approval and after patients or guardians gave their informed consent. Myocytes were isolated from the right or left atrial appendage or from ventricular septal tissue. Tissue samples were transported to the laboratory in a solution containing (mM) 120 NaCl, 4.4 KCl, 1.5 MgCl2, 0.33 NaH2PO4, 7.5 glucose, 16 taurine, 5 pyruvate, 15 2.3-butanediol monoxime (BDM), and 5 HEPES, pH 7.4 adjusted with NaOH. Tissue specimens were diced into cubic pieces (1 mm3), placed in flasks containing 10 ml of the above solution plus 1.5 mg/ml collagenase (Type 1, Sigma), and gently stirred with a magnetic bar. The supernatant was removed every 40 min and substituted with fresh enzyme-containing solution. The third and fourth supernatants were collected, and cells were pelleted with gentle centrifugation (253 g for 3 min) and resuspended in high-K+ storage solution containing (mM) 45 KCl, 70 K-glutamate, 3 MgSO4, 15 KH2PO4, 16 taurine, 10 HEPES, 0.5 EGTA, and 10 glucose (pH 7.38). The cell suspension was kept at room temperature for at least 1 h before transfer to Eagle’s minimum essential medium (Sigma) containing 1 mM Ca2+.

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Human coronary artery endothelial cells and smooth muscle cells. Human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) were obtained from Cambrex (East Rutherford, NJ) and kept according to the manufacturer’s recommendations. The HCAEC originated from a 32-year-old Caucasian woman and the HCASMC from a 59-year-old Caucasian man. These primary cells have been cryopreserved and are sold at their third passage. To avoid any alterations produced by long-term culturing, we patch-clamped the cells in their fourth to eighth passages.

Electrophysiological recording. Cells, on 15-mm coverslips, were placed in a bath on the stage of an inverted microscope. Single-channel recordings were made at room temperature in the inside-out patch-clamp mode, and data were obtained with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) and standard patch-clamp techniques. Patch pipettes were pulled from borosilicate glass (OD 1.5 mm; Sutter Instrument) and had 6- to 8-MΩ resistances when filled with pipette solution. After formation of gigaseals (usually >5 GΩ), patches were excised. Inside-out patch currents were filtered (Bessel response; cutoff frequency −3 dB at 1 kHz) and digitized at 5 kHz and stored on a computer hard disk with pCLAMP software (Clampex 9.0, Axon Instruments). The holding potential was −80 mV (pipette potential was +80 mV). The command voltage was changed (steps of 2-s duration) to +160, +140, +120, or +100 mV, repeated at an interval of 2 s. Once channel activity was observed, it was recorded at a holding potential of −60 mV with voltage steps (−1- to 4-s duration) between +120 and −100 mV or alternatively by continuous gap-free recordings at a given holding potential. Recordings were performed at room temperature (21–25°C).

Recording solutions. The standard pipette solution (150 mM Cl− solution) contained (in mM) 150 KCl, 1 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4. For estimating the Cl− effects of extracellular nucleotides, 74 or 30 mM Cl− solutions was added to the pipette solution. To examine the effects of extracellular nucleotides, −0.01–0.5 mM K2-ATP or 0.1 mM ADP, AMP, ATPγS, and UTP salts was added to the pipette solution as needed. The bath solution used for cardiac myocytes contained (mM) 150 KCl, 0.5 EGTA, 5 HEPES, 0.25 ATP, and 10 glucose, pH 7.2. For HCAEC or HCASMC, the bath solution contained (mM) 150 KCl, 5 HEPES, 1 MgCl2, 10 glucose, and 0.1 ATP, pH 7.4. ATP was included in the bath to inhibit ATP-sensitive K+ (KATP) channels in cardiomyocytes. Of note, the same bath solution would become “cytosolic solution” to the inside-out patch once the patches were excised from cell-attached patches. The liquid junction potentials of solutions were calculated (pCLAMP software), and data were corrected for the liquid junction potential when necessary.

Data analysis. pCLAMP 9.0 (Axon Instruments) software was used to construct amplitude histograms, which were used to determine open and closed dwell times. Single-channel current amplitudes were determined from all-points amplitude histograms fitted with a sum of two Gaussian distributions. The open probability of the channel (Po) was defined as the ratio of open channel area relative to the total area under the curve. Dwell time distributions were constructed from 0.5- to 3-min continuing recordings with logarithmic binning. Events were detected with a 50% threshold criterion. Open and closed dwell time distribution histograms of events were fitted by exponential probability density functions and the maximum-likelihood method. Origin7.0 (OriginLab, Northampton, MA) was used for further analysis. Unless otherwise noted, parameter values are given as means ± SE.

RESULTS

ORCC in human cardiovascular tissue. In our initial experiments, the ORCC was recorded in human atrial and ventricular myocytes. In 6.8% of patches excised from atrial cells (10 of 148, in the presence of 0.25 mM ATP in the bath solution) we found a robust outward rectifying current similar to the atrial ORCC reported by others (5, 9, 10). ORCCs were also found in ventricular (septal) myocytes (2 of 18 patches from 1 patient). After patch excision, channel activity was recorded that could last for from several minutes to half an hour before rundown occurred. Figure 1 depicts typical recordings of
ORCC. A plot of the unitary current amplitude as a function of voltage (Fig. 1B) reveals the ORCC to be outward rectifying under our experimental conditions. The slope conductances at +60 mV and −60 mV were 97.2 ± 2.45 pS (n = 10) and 22.5 ± 2.50 pS, respectively, as calculated by linear regression analysis of the single-channel currents measured between 40 and 80 mV and between −40 and −80 mV. The ratio of conductance at +60 to that at −60 mV is ~4–5, which is characteristic of a channel with outward rectifying properties reported previously in rabbit and human atrial myocytes (5, 9, 10). ORCC gating was voltage independent, since $P_o$ of the channel was near maximal over the entire voltage range examined (Fig. 1C). Channels with identical properties were also present in 8.7% (6 of 69, 0.1 mM bath ATP) of isolated patches from HCAEC and 26.9% (7 of 26) patches of HCASMC.

Since gating of ORCC in endothelial cells was voltage independent, we further investigated the properties of ORCCs in cultured endothelial cells, which are obtained more readily than human atrial or ventricular myocytes. To establish that ORCC is indeed a Cl$^−$ channel, we reduced the extracellular (pipette) Cl$^−$ concentration and found the outward current to be reduced, which resulted in a shift of the I-V relationship to more positive voltages (Fig. 3). Plotting the reversal potential as a function of the Cl$^−$ concentration (pipette Cl$^−$ concentration) resulted in a linear relationship with a slope of ~47 mV per decade, which is close to the value calculated from the Nernst equation for a Cl$^−$ electrode, demonstrating that ORCC is highly selective for Cl$^−$.

Modulation of ORCC by nucleotides. Since intracellular ATP is necessary for the activation of VRAC (22), most of our recordings were made by including ATP in the bath solution. When omitting ATP from the bath solution, we found a substantial reduction in the number of patches in which ORCC was present. For example, the percentage of patches from endothelial cells containing ORCC decreased from 8.7% (6 of 69 patches; $P < 0.05$ by $y^2$-test with Yates correction). We did not pursue the mechanisms involved in the sensitivity of ORCC to cytosolic ATP. It is unlikely to be due solely to phosphorylation events, since ORCC was recorded in 6.7% of atrial patches under Mg$^{2+}$-free conditions (which should prevent phosphorylation).

We next examined the effects of extracellular ATP by including it in the pipette solution but not in the bath solution. The inclusion of extracellular ATP (0.1 mM) by itself did not...
enhance the incidence of patches containing ORCC (1 of 61 patches in the absence of bath ATP, which is not different from 7 of 338 when ATP was absent on both sides of the membrane; P < 0.05). Furthermore, there was no additive effect of extracellular ATP to that of intracellular ATP. In the presence of 0.1 mM intracellular ATP, the incidence was 8.7% (6 of 69) in the absence of extracellular ATP and 11.8% (20 of 169; P < 0.05) when ATP (0.05 mM) was included in the pipette solution. These data are summarized in Table 1. Overall, these data suggest that intracellular, but not extracellular, ATP increases the number of active channels in membrane patches.

Intracellular ATP had no effect on ORCC gating, since P_o was unaffected by 2 mM ATP in the bath solution (n = 4; data not illustrated). In contrast, in recordings with ATP in the pipette solution (extracellular ATP), P_o was substantially and dose-dependently reduced (Fig. 4). The half-maximal inhibition concentration (IC_50) was 0.12 mM ATP at 80 mV (Fig. 4, inset a). The reduction in P_o by extracellular ATP was voltage dependent, decreasing with progressively positive voltages (Fig. 4, inset b). The degree of extracellular ATP-induced inhibition of P_o was similar in endothelial cells and atrial myocytes. For example, at +80 mV, 0.1 mM extracellular ATP reduced P_o from 0.98 ± 0.01 (n = 6) to 0.56 ± 0.04 (n = 4; P < 0.05) in endothelial cells and from 0.99 ± 0.02 (n = 7) to 0.55 ± 0.05 (n = 4; P < 0.05) in atrial cells. In the presence of extracellular ATP, channel openings were interrupted by rapid closing events (Fig. 5). Open time distributions were best represented as a single exponential function, both in the absence and presence of extracellular ATP. Although a sum of two exponentials produced a slightly better data fit in some cases, we used a single exponential function to represent the closed time distributions. Without extracellular ATP, the mean open time was 61.2 ± 5.79 ms and the mean closed time was

![Fig. 3. Dependence of single-channel current on Cl^- concentration. A: typical single-channel current was recorded from endothelial cells with pipette solution containing 150 (top), 74 (middle), and 30 (bottom) mM Cl^- (Cl^- was replaced by equimolar substitution with glutamate, and holding potential was +80 mV; these recordings were made in different patches). Note the flickery nature of channel opening due to the presence of 50 μM ATP in the pipette solution (cf. Fig 4; also see text). Insets are all-point histograms of current amplitudes with a bin width of 0.1 pA. B: average current-voltage (I-V) curves of single-channel current at concentrations of 150 (n = 10), 74 (n = 5), or 30 (n = 4) mM Cl^- in the pipette. Liquid junction potentials were calculated to be 0, 5.3, and 9.0 mV for 150, 70, and 30 mM Cl^- solutions, respectively. Data are corrected for the liquid junction potential. C: plot of the reversal potentials (V_r) of data obtained in B as a function of pipette Cl^- concentration ([Cl]_p). Data points were fitted to a linear function, resulting in a slope of 47 mV per decade.](http://ajpheart.physiology.org/)

Table 1. Effect of intracellular ATP on number of patches containing ORCC in endothelial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP_o, mM</th>
<th>ATP_i, mM</th>
<th>Patches</th>
<th>Incidence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>7/338</td>
<td>2.5</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0.1</td>
<td>6/69*</td>
<td>8.7</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0.1</td>
<td>1/61</td>
<td>0.1</td>
</tr>
<tr>
<td>D</td>
<td>0.1</td>
<td>0.1</td>
<td>20/169*</td>
<td>11.8</td>
</tr>
</tbody>
</table>

ORCC, outwardly rectifying Cl^- channel; [ATP]_o, [ATP]_i, concentrations of ATP in the pipette and bath solutions, respectively, which correspond to extracellular and intracellular ATP concentrations in the inside-out patch clamp configuration. *P < 0.05 compared to the first group that had no ATP on either side of the membrane (χ²-test). There were no statistically significant differences between groups B and D.
Thus extracellular ATP concentration-dependently reduced mean open time and concomitantly increased mean close time (Fig. 5, bottom), suggesting that the open state of ORCC is destabilized by extracellular ATP.

Next we investigated the effects of other nucleotides by including them in the pipette solution. ADP or AMP (0.1 mM each) had weak effects on ORCC activity (Fig. 6). The order of block was ATP > ADP > AMP. ATP hydrolysis does not appear to be required, since a nonhydrolyzable ATP analog, ATPγS, also strongly decreased ORCC activity and UTP caused a similar degree of block as ATP (Fig. 6). The voltage-dependent block of ORCC and the block with sequence of ATPγS > ATP = UTP > ADP > AMP strongly suggest that a negative charge in the nucleotide is an important determinant in channel block. However, it is also possible that P2 receptor activation may be involved. To examine this possibility, preliminary experiments were performed by including 0.5 mM suramin, a nonspecific P2 receptor antagonist, in the pipette solution. By itself, however, suramin strongly reduced ORCC activity (Pₒ at +80 mV was reduced from 0.99 to 0.26 ± 0.03; n = 2). In addition, the P2x receptor antagonist pyridoxal phosphate azophenyl-2,4-disulfonic acid (PPADS, 0.1 mM) and the P2y receptor antagonists 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS, 0.2 mM) and reactive blue 2 (RB, 1–100 μM) also strongly inhibited ORCC activity. These findings precluded us from investigating the involvement of purinergic receptors in the modulation of ORCC by extracellular ATP. Future studies using other approaches (such as using purinergic receptor-knockout animals) may be needed to address this question.

**DISCUSSION**

Cl⁻ channels are present in the plasma membranes of many cells and play potentially important roles in the control of cell volume, intracellular pH, and membrane potential. Several
different Cl⁻ conductances have been identified in the sarcolemma of cardiac myocytes isolated from various animal species and areas of the heart (17). Previously, a cardiac ORCC was identified in rabbit atrial myocytes (10) and in human atrial myocytes (5). In this study, we found ORCC not only present in human atrial myocytes but also present in septal ventricular myocytes as well as in primary human coronary smooth muscle and coronary arterial endothelial cells. Surprising, ORCCs in those different cardiac tissues are similar in terms of their outward rectifying properties, I-V curves, and modulation by ATP, which suggests that ORCCs are ubiquitously expressed in the cardiovascular system and may have similar molecular composition. The conductance of cardiovascular ORCCs we recorded here was ~90–100 pS at positive potential range (+40 to +80 mV), which is slightly larger than that of atrial ORCCs reported previously (~60–76 pS) (5, 10) and much larger than noncardiac ORCCs (40–50 pS) (1, 13, 26). Thus there appears to be tissue-specific diversity in the properties of ORCC. Other possible reasons for the observed differences might include the use of different regions of the I-V curve for calculating the slope conductance or the fact that higher pipette Cl⁻ concentrations were used in our study. The ORCCs recorded in our studies had biophysics properties and sensitivity to glibenclamide similar to those in cardiomyocytes and non-cardiomyocytes.

In previous studies, ORCCs were suggested to be partially responsible for VRAC in atrium (5, 9). Our result also supports this conclusion. First, the wide distribution of ORCC in the...
heart also highly suggests that ORCC is involved in the formation of VRAC, since VRAC is a ubiquitously expressed Cl− channel. Second, we found at the single-channel level that intracellular ATP increases the incidence of patches containing ORCC. This observation is consistent with previous reports suggesting that the activation and rundown properties of macroscopic VRAC depend on intracellular ATP levels (22, 23). Furthermore, activation of volume-activated Cl− current by hypotonic cell swelling in guinea pig atrial cells was also shown to be dependent on nonhydrolyzable intracellular ATP (24). Swelling-induced Cl− currents are affected by phosphorylation events [for example, by tyrosine kinases (28)], and it may be possible that ATP may be affecting VRAC through such a mechanism. However, phosphorylation events are unlikely to be the sole factor involved (since in our experiments ATP also increased channel availability under Mg2+ -free conditions). An untested possibility is that intracellular ATP may enhance ORCC opening by directly activating CFTR, since ATP is a well-known regulator of CFTR and recent evidence suggests that CFTR is a regulator of ORCC (19, 20). Third, we found extracellular ATP to inhibit ORCC activity in human cardiac tissue by decreasing its $P_o$. Block of a native volume-activated Cl− current by extracellular ATP has also previously been demonstrated in cardiac myocytes from the guinea pig atrium (8). In addition, heterologously expressed ClC-3 channels, which resemble native volume-activated Cl− currents in most mammalian cells, are also blocked by extracellular ATP. In our experiments, the rank order of potency was ATP > ADP > AMP, which is again similar to the order previously reported for VRAC in epithelial and endothelial cells (16, 30). Extracellular nucleotides caused a flickery block of the channel and reduced $P_o$ by increasing the closed dwell time and decreasing the mean open dwell time. ATP hydrolysis at the extracellular site appeared not to be required for channel inhibition since ATPγS, a nonhydrolyzable analog of ATP, caused an even more pronounced block than ATP. Thus the number of phosphate groups appears to be a critical determinant for inhibition. Of note, the voltage dependence of block by
nucleotides is different for ORCC and VRAC, although with similar rank order of potency. Extracellular ATP blocked ORCC with a linear voltage dependence at positive voltages (approximately +20 to +100 mV), while block of VRAC by extracellular ATP was described as bell shaped, decreasing at voltages positive to +40 mV (16). This may indicate that other factors may be involved in regulation of VRAC by extracellular ATP.

Earlier studies on ORCC in human airway epithelial cells demonstrated that extracellular ATP increases ORCC $P_e$ through activation of the P$_2$ receptor pathway (25, 29). We did not observe such an increase. On the contrary, we found extracellular ATP to inhibit ORCC activity in human cardiac tissue by decreasing its $P_e$. The reasons for this apparent discrepancy are not clear, but it may be attributable to the different tissues or species examined or to possible differences in the recording conditions. Interestingly, P$_2$ antagonists such as suramin, PPADS, DIDS, and RB also block the ORCC; however, the nature of inhibition is not clear at present (it could be either antagonistic or nonspecific) and may be worthy of future studies.

Other than being a component of the VRAC, the physiological role of ORCC in the cardiovascular system is not clear at this time. However, the ubiquitous presence of ORCC in the cardiovascular system may point to important functions. Our data demonstrate that ORCC is expressed in cardiac myocytes of patients from 3 mo to 63 yr of age and is found in several cell types of the human heart. The channel may therefore have an important role under physiological and/or pathophysiological conditions. Furthermore, the antagonistic effects of intracellular and extracellular ATP on ORCC may also be of significance. Of note, the number of patches containing ORCCs was higher in patient samples than in control hearts.

In summary, we identified an outwardly rectifying Cl$^-$ channel (ORCC) that is widely distributed in human cardiac tissue. ORCCs from those tissues were upregulated by intracellular ATP but inhibited by extracellular ATP. Thus ATP may play an important role in regulation of Cl$^-$ transport under some pathological conditions.

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REFERENCES


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