

Pharmacological Properties and Physiological Function of a P2X-Like Current in Single Proximal Tubule Cells Isolated from Frog Kidney

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Abstract Although previous studies have provided evidence for the expression of P2X receptors in renal proximal tubule, only one cell line study has provided functional evidence. The current study investigated the pharmacological properties and physiological role of native P2X-like currents in single frog proximal tubule cells using the whole-cell patch-clamp technique. Extracellular ATP activated a cation conductance ($P2X_f$) that was also Ca^{2+} -permeable. The agonist sequence for activation was $ATP = \alpha\beta\text{-MeATP} > \text{BzATP} = 2\text{-MeSATP}$, and $P2X_f$ was inhibited by suramin, PPADS and TNP-ATP. Activation of $P2X_f$ attenuated the rundown of a quinidine-sensitive K^+ conductance, suggesting that $P2X_f$ plays a role in K^+ channel regulation. In addition, ATP/ADP apyrase and inhibitors of $P2X_f$ inhibited regulatory volume decrease (RVD). These data are consistent with the presence of a P2X receptor that plays a role in the regulation of cell volume and K^+ channels in frog renal proximal tubule cells.

Keywords Purinergic receptor physiology in epithelia · Renal physiology · Ion channel · Potassium ion channel · Volume regulation in epithelial cells

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Introduction

P2 purinoceptors are a class of ATP-activated receptors that play a critical role in a variety of cellular processes in both electrically excitable and nonexcitable cells. They are subdivided into two distinct classes, P2X and P2Y receptors (Burnstock and Kennedy 1985; North and Barnard 1997). P2X receptors are ATP-gated, nonselective, Ca^{2+} -permeable cation channels that, on activation, allow extracellular Ca^{2+} to enter the cell, leading to a rise in intracellular Ca^{2+} . The P2Y receptors are G protein-coupled. On activation, some P2Y receptors cause a rise in intracellular Ca^{2+} , via the release of Ca^{2+} from intracellular stores.

At the molecular level a number of mammalian P2X and P2Y receptors have been identified, $P2X_{1-7}$ and $P2Y_{1,2,4,6,11-14}$, with only two amphibian P2X receptors identified, $P2X_4$ (Juranka et al. 2001) and a $P2X_5$ -like current (Jensik et al. 2001). These receptors can act as homomeric channels and form heteromeric channels. To date, in heterologous systems, combinations of a number of P2X receptors have been observed, including $P2X_{2/3}$, $P2X_{1/5}$ and $P2X_{4/6}$ (Le et al. 1998, 1999; Radford et al. 1997; Torres et al. 1999). There is also evidence for the presence of $P2X_{2/3}$ heteromers in rat nodose neurons (Lewis et al. 1995). A more recent study has provided evidence for $P2X_{4/7}$ receptors (Guo et al. 2007). The properties of these heteromers are determined by their receptor composition, with properties taken from both receptor types. This means that heteromeric channels demonstrate very different properties from the single cloned receptors.

Both P2X and P2Y family members are found in the kidney, with the majority of work on renal P2 receptors to date focusing on their role in the distal tubule and collecting duct. Previous work has demonstrated that both

P2X and P2Y receptors regulate the activity of the epithelial Na^+ channel (ENaC). P2Y₂ receptors inhibit ENaC function (Pochynyuk et al. 2008), while basolateral P2X₄ and heteromeric P2X_{4/6} have been shown to activate ENaC (Wildman et al. 2008; Zhang et al. 2007). In inner medullary collecting duct cells there is evidence for a role for P2X₁, P2X₃, P2X₄, P2Y₁ and P2Y₂ (McCoy et al. 1999; Xia et al. 2004) in regulating Na^+ and Cl^- transport. In addition, P2 receptors regulate aquaporin-2-mediated water reabsorption and K^+ channel activity in the collecting duct (Lu et al. 2000; Wildman et al. 2009). In the thick ascending limb P2 receptors also play a regulatory role, with P2Y₂ and an as yet unidentified P2X receptor involved (Jensen et al. 2007; Silva and Garvin 2009). P2Y₂ receptors are also thought to be important in macula densa cell signaling (Liu et al. 2002) and in the modulation of apoptosis of human mesangial cells (Solini et al. 2007). In the proximal tubule a significant body of work has concentrated on P2Y receptors, with molecular and functional approaches indicating the presence of P2Y₁, P2Y₂, P2Y₄ and P2Y₆ (Bailey 2004; Bailey et al. 2001; Cha et al. 1998; Chan et al. 1998; Lee et al. 2005). These P2Y receptors are thought to play an important role in regulating Na^+ -glucose transport activity, HCO_3^- reabsorption and the regulation of basolateral Cl^- channels (Bailey et al. 2001; Bouyer et al. 1998; Lee et al. 2005). In contrast, very few studies have identified P2X receptors at either the molecular or the functional level in proximal tubule. The few studies completed have used cell lines and primary cultures and provide evidence for the expression of P2X₁, P2X₄, P2X₅ and P2X₆ (Filipovic et al. 1998; Leipziger and Unwin 2003; Takeda et al. 1998). At a functional level, only studies in LLC-PK₁ cells have shown ATP-activated, P2X-mediated currents (Filipovic et al. 1998). In these cells, the pharmacological properties of the current were consistent with P2X₂, although RT-PCR identified a fragment that closely resembled rat P2X₁. To date, no study has shown evidence for the existence of functional P2X receptors in native proximal tubule cells. Therefore, the aim of the current study was to examine the physiological function and pharmacological properties of an ATP-activated current in freshly isolated frog single proximal tubule cells.

Methods

Cell Isolation

Single proximal tubule cells were isolated from *Rana temporaria* using an enzyme digestion technique (Hunter 1989). Frogs were killed by stunning, and the brain and spinal cord were destroyed prior to removal of the kidneys, in accordance with U.K. legislation. Proximal tubule cells

were identified by their “snowman” appearance (Robson and Hunter 1994c).

Cell Length Experiments

Cell length was measured using two different techniques that utilize changes in light intensity at the cell membrane/bath interface. The first technique used a photodiode array-based system as described previously (Mounfield and Robson 1998), while the second technique used a digital camera-based system (Soft Cell; Cairn Research, Kent, UK). Cells were initially superfused with frog Ringer that contained (in mM) 50 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES (titrated to pH 7.4 using NaOH) and 89 mannitol. Hypotonic shock was then induced by the removal of 40 mM mannitol. This was repeated in unpaired cells in the presence of varying P2X receptor antagonists (camera-based system) or in the presence of ATP/ADP apyrase (array-based system), which breaks down ATP. Thus, if ATP release is important in volume regulation, then ATP/ADP apyrase should inhibit the regulatory response. Antagonist or apyrase was present in both control and hypotonic solutions. All test solutions were compared to day-matched controls.

Patch Experiments

Approximately 20 μl of the cell suspension was placed in a Perspex bath on the stage of an inverted microscope (IX70; Olympus, Tokyo, Japan). Standard patch-clamp techniques were employed to investigate whole-cell currents (Hamill et al. 1981), with voltage protocols driven from a computer equipped with a Digidata interface (Axon Instruments, Foster City, CA). Data were obtained and analyzed using pClamp (Axon Instruments). Recordings were made using a List EPC-7 amplifier (HEKA, Lambrecht, Germany). On achieving the whole-cell configuration via the basolateral aspect of the cell, currents were saved directly onto the hard disk of the computer following low-pass filtering at 5 kHz. Microsoft (Redmond, WA) Excel 2000 was used to determine average steady-state currents at each potential. Cell area was calculated from the capacity transients seen in response to a 20-mV potential step, with membrane capacitance assumed to be 1 $\mu\text{F}/\text{cm}^2$. Except where stated, the pipette contained a high- Na^+ solution (in mM) 100 NaCl, 2 MgCl₂, 0.5 EGTA and 10 HEPES (titrated to pH 7.4 with NaOH) and the bath contained (in mM) 100 NaCl, 0.5 CaCl₂, 0.5 MgCl₂ and 10 HEPES (titrated to pH 7.4 with NaOH). In experiments investigating the properties of the ATP-activated current, the total ATP added to the extracellular solution was adjusted to give a constant free ATP concentration between control and test conditions (Maxchelator, maxchelator.stanford.edu). For higher

concentrations of agonists, osmolality was maintained by substitution of mannitol.

Two different voltage-clamp approaches were used. In one set of experiments clamp potential was held constant at -100 mV and changes in current at this potential were recorded over time. At various time points potential was ramped to between -100 and $+20$ mV. In the second set of experiments whole-cell potential was clamped at -40 mV and then stepped to between $+20$ and -100 mV in -20 mV steps. The reversal potential (V_{rev}) of currents obtained using this voltage protocol was determined using polynomial regression analysis. The ATP-activated conductance (G_{ATP}) was taken over the potential range -100 to -20 mV. For K^+ current studies, outward (G_{out}) and inward (G_{in}) chord conductances were calculated from outward and inwardly directed currents, respectively.

Effect of ATP and BzATP

Patches were exposed to 100 μM , 200 μM , 500 μM and 2 mM $3'$ - O -(4-benzoyl)benzoyl ATP (BzATP) and ATP. The order of exposure to the two agonists was varied to ensure that there was no effect of desensitization. To examine possible desensitization, whole-cell patches were exposed to an agonist (either 2 mM BzATP or 500 μM ATP) three times. The dose response to these agonists was determined using the following bath solution (in mM): 85 NaCl, 0.5 CaCl_2 , 0.1 MgCl_2 , 25 mannitol and 10 HEPES (titrated to pH 7.4 with NaOH). Cells were exposed to either ATP or BzATP (10 , 8 , 4 , 2 , 1 and 0.5 mM).

Agonist Potency and Effect of Antagonists

Patches were exposed to 500 μM ATP, followed by 500 μM of a second agonist, either BzATP, 2-methylthio ATP (2-MeSATP) or α,β methylene ATP ($\alpha\beta$ -MeATP). To examine the effect of antagonists, patches were exposed to 500 μM ATP alone, then ATP in the presence of one of the following P2X receptor antagonists: suramin, $2',3'$ - O -(2,4,6-trinitrophenyl)-ATP (TNP-ATP) or pyridoxal-5-phosphate-6-azophenyl- $2',4'$ -disulfonic acid (PPADS). The effect of the P2X₇ antagonists brilliant blue G (BBG) (Jiang et al. 2000) and KN-62 (Humphreys et al. 1998) were examined by exposing patches to 2 mM BzATP and then BzATP in the presence of the antagonist.

Current Properties and Relative Permeability of ATP-Activated Current

Whole-cell patches were obtained with the standard high- Na^+ pipette solution. The bath contained (in mM) 100 NaCl, 0.5 CaCl_2 , 0.1 MgCl_2 and 10 HEPES (titrated to pH 7.4 with NaOH). Current activation was achieved using 2 mM ATP.

To examine the effect of ivermectin, an antiparasitic agent known to enhance activation of P2X₄ (Priel and Silberberg 2004), whole-cell patches were obtained with a bath solution containing (in mM) 20 NaCl, 0.5 CaCl_2 , 0.1 MgCl_2 , 160 mannitol and 10 HEPES (titrated to pH 7.4 with NaOH). The effect of 10 μM ivermectin was determined in paired cells, with patches exposed to 2 mM ATP initially and then after incubation with ivermectin for 1 min.

To determine the cation to anion permeability ratio, a dilution protocol was used. ATP-activated currents were recorded in paired cells in both 100 and 20 mM NaCl. Osmolality was maintained by the addition of 160 mM mannitol to the 20 mM solution. The junction potential shift induced by reducing bath NaCl fivefold was measured using a flowing 3 M KCl reference electrode, and all 20 mM NaCl V_{rev} values were corrected for this value. The monovalent cation permeability ratio was determined in paired cells by substituting 100 mM NaCl with either RbCl, CsCl or LiCl. To determine the relative permeability for Na^+ to NMDG^+ , whole-cell patches were obtained with 100 mM NaCl, 0.5 mM EGTA and 10 mM HEPES (titrated to pH 7.4 using NaOH) in the pipette and 100 mM NMDG-Cl and 10 mM HEPES (titrated to pH 7.4 using NMDG^+) in the bath. The ATP-activated current was determined (addition of 4 mM ATP to the bath). To determine the Ca^{2+} to Na^+ permeability ratio, the extracellular solution was then exchanged for one that contained 5 mM CaCl_2 , in addition to NMDG-Cl and HEPES. For all permeability ratio calculations the Goldman-Hodgkin-Katz voltage equation was used, with a modified form utilized in the Ca^{2+} to Na^+ calculation (Laycock et al. 2009).

Effect of Extracellular Cations

The bath contained (in mM) 100 NaCl and 10 HEPES (titrated to pH 7.4 with NaOH). Patches were exposed to 2 mM ATP under the control circumstance and then in the presence of either 100 μM Zn^{2+} or 50 μM Cu^{2+} . In Ca^{2+} experiments the effect of ATP was examined with either 0.5 (control) or 2 (test) mM Ca^{2+} in the bath. The effect of extracellular pH was examined using the standard bath solution, with patches exposed to 2 mM ATP at pH 7.4 and either pH 6.3 (addition of HCl) or pH 8.3 (addition of NaOH).

Effect of P2X Activation on Whole-Cell K^+ Conductance

To determine the effect of activation of the frog P2X receptor on K^+ channels, whole-cell K^+ currents were measured in the absence and presence of ATP. The pipette contained (in mM) 100 KCl, 2 MgCl_2 , 10 HEPES (titrated to pH 7.4 with KOH) and 0.25 EGTA as well as 25 units/ml

alkaline phosphatase, which was included to reduce K⁺ channel rundown. The bath contained (in mM) 92 NaCl, 3 KCl, 1 CaCl₂, 0.5 MgCl₂, 10 HEPES (titrated to pH 7.4 with NaOH) and 20 mannitol. On achieving the whole-cell configuration, 1 mM quinidine was added to the bath and the current sensitive to quinidine was taken as the magnitude of the K⁺ currents. Quinidine has previously been shown to inhibit two K⁺-selective currents in frog renal proximal tubule cells (Robson and Hunter 1997). The quinidine-sensitive current was then determined again after 5 min. To examine the effect of P2X receptor activation, whole-cell patches were obtained as described previously. However, after the first exposure to quinidine and subsequent wash, cells were exposed to 4 mM ATP. ATP was left in the bath for 5 min, and the quinidine-sensitive current was determined after this time.

Chemicals and Solutions

The osmolality of all solutions was measured (Roebeling osmometer) and adjusted to within 1 mosmol/kg water of 215 mosmol/kg water with water or mannitol as appropriate. Chemicals were obtained from Sigma (St. Louis, MO) and were of analytical grade.

Statistics

Results are given as means \pm 1 SEM, with the number of experiments in parentheses. Except where stated in the text, significance was tested using paired Student's *t*-test. Additional tests included unpaired Student's *t*-test, ANOVA, Fisher's exact probability test and correlation coefficient as appropriate. For all, significance was assumed at the 5% level.

Results

Effect of ATP and BzATP

Both ATP and BzATP increased whole-cell currents at -100 mV, although ATP gave a greater increase compared to BzATP. ATP increased whole-cell currents at all concentrations tested ($P \leq 0.001$ for all). BzATP increased whole-cell currents at 2 mM ($P = 0.004$) and 500 μ M ($P = 0.0004$) but was without effect at 200 μ M ($P = 0.267$) and 100 μ M ($P = 0.192$). Figure 1a, b shows typical traces from cells exposed to 500 μ M ATP. Mean agonist-activated currents are shown in Fig. 1c. At all concentrations the response to ATP was greater than the response to BzATP (unpaired Student's *t*-test). The BzATP and ATP activated current did not demonstrate desensitization. The 2 mM BzATP-activated currents were

-12.5 ± 1.67 , -16.0 ± 4.52 and -11.51 ± 1.92 pA ($n = 13$) for first, second and third exposures, respectively. The mean 500 μ M ATP activated currents were -10.6 ± 2.96 , -8.77 ± 1.78 and -9.08 ± 1.23 pA ($n = 13$) for first, second and third exposures, respectively. There was no significant difference between these: $F_{2,36} = 0.69$ and $F_{2,36} = 0.23$, for BzATP and ATP, respectively (ANOVAs). There was also no correlation between current activation and exposure number ($r^2 = 0.04$ and $r^2 = 0.08$).

ATP activated the whole-cell current in a dose-dependent manner (Fig. 1d). Half-maximal activation was observed with 2.77 ± 0.24 mM ATP ($n = 9$), with a Hill coefficient of 3.89 ± 0.57 and maximal current of -30.1 ± 3.68 pA ($r^2 = 0.992 \pm 0.002$). BzATP also activated the currents in a dose-dependent manner. Half-maximal activation was observed with 4.00 ± 0.38 mM BzATP ($n = 7$), with a Hill coefficient of 2.00 ± 0.07 and maximal current of -32.1 ± 2.31 pA ($r^2 = 0.997 \pm 0.001$). The ATP concentration required for half-maximal activation was significantly smaller than the half-maximal concentration of BzATP ($P = 0.01$, unpaired Student's *t*-test). There was no significant difference between the maximal current activated by either agonist ($P = 0.62$, unpaired Student's *t*-test).

Agonist Potency and Effect of Antagonists

At 500 μ M, ATP, BzATP, 2-MeSATP and $\alpha\beta$ -MeATP all increased whole-cell currents ($P \leq 0.0001$). In paired cells the effect of ATP on whole-cell current was greater than the effect of either BzATP ($n = 12$, $P = 0.003$) or 2-MeSATP ($n = 24$, $P < 0.0005$) (Fig. 2). In contrast, ATP and $\alpha\beta$ -MeATP increased currents by comparable amounts ($n = 13$, $P = 0.30$) (Fig. 2). The agonist potency sequence was ATP = $\alpha\beta$ -MeATP > BzATP = 2-MeSATP. In the presence of 100 μ M suramin ($n = 16$), 30 nM TNP-ATP ($n = 16$) or 10 μ M PPDAS ($n = 21$), the ATP-activated current was inhibited by 30%, 44% and 34%, respectively ($P < 0.008$ for all) (Fig. 3). The inhibition by suramin and PPDAS was reversible, but that by TNP-ATP was not. It has previously been demonstrated that inhibition by PPDAS can be increased after incubation for 10 min. However, in paired cells the ATP-activated current in the presence of PPDAS at 1 min, -9.00 ± 1.82 pA ($n = 9$), was not significantly different from that at 10 min, -10.2 ± 4.12 pA ($n = 9$, $P = 0.65$). PPDAS (100 μ M) inhibited the ATP-activated current by 65%, -11.2 ± 1.91 pA ($n = 9$) vs. -3.94 ± 1.12 pA, in the absence and presence of PPDAS, respectively ($P = 0.01$). On wash the response to ATP recovered, -11.1 ± 1.64 pA. P2X₇ antagonists were without effect on BzATP-activated currents. BzATP increased whole-cell current by -22.3 ± 6.86 pA ($n = 6$) and -21.9 ± 8.41 pA in the absence and presence of BBG,

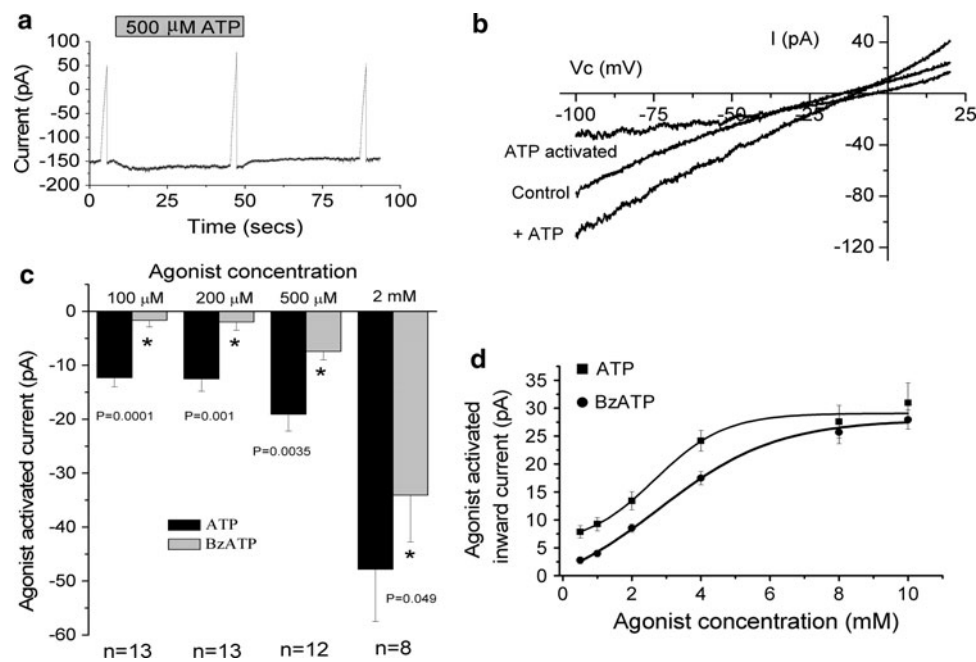


Fig. 1 Effect of different concentrations of ATP and BzATP. Clamp potential was held at -100 mV. At three time points (control, plus ATP and wash) a ramp protocol was run between -100 and $+20$ mV. **a** A typical trace (current in picoamperes against time in seconds), showing the effect of 500 μM ATP. Solid bar indicates when ATP was present in the bath. **b** Typical I - V traces showing the current recorded under the control circumstance, in the presence of 500 μM ATP and the ATP-activated current. V_c is the command voltage.

c The mean agonist-activated current in response to different concentrations of ATP and BzATP in paired cells. * Significant difference from ATP, with corresponding P values given below each set of data. n , number of patches. **d** Dose response to ATP (filled square) and BzATP (filled circle). Lines through data points are the best fit to the Hill equation, $r^2 = 0.996$ and 0.998 for ATP and BzATP, respectively

respectively ($P = 0.91$). In the second set of experiments, BzATP increased whole-cell current by -32.0 ± 7.29 pA ($n = 6$). In the presence of KN-62 this was unchanged, -27.8 ± 7.24 pA ($P = 0.27$).

Current Properties and Relative Permeability of ATP-Activated Current

Figure 4a shows typical traces obtained in the absence and presence of 2 mM ATP. Addition of ATP to the bath increased whole-cell currents (Fig. 4) ($P < 0.001$). The inward chord conductance under the control circumstance was 30.2 ± 2.66 μS/cm² ($n = 14$). On addition of ATP this increased to 39.7 ± 3.18 μS/cm², a mean increase of 9.53 ± 0.90 μS/cm². The ATP-activated point conductance from these 14 cells is shown in Fig. 4c. Conductance and potential demonstrated a significant negative correlation ($r^2 = -0.86$, $P < 0.05$), indicating that the ATP-activated current demonstrated inward rectification. The ATP-activated current was potentiated by 1-min incubation with 10 μM ivermectin. Initially, ATP increased the current at -100 mV from -710 ± 216 to -809 ± 232 pA ($n = 7$). However, after incubation of the same patches in ivermectin, the current increased from -657 ± 220 to -785 ± 239 pA ($n = 7$). The mean ATP-activated current

in the presence of ivermectin, which enhances the activation of P2X₄, was significantly greater, -98.1 ± 23.2 vs. -127 ± 23.1 pA ($n = 7$, $P = 0.025$), in the absence and presence of ivermectin, respectively.

With 100 mM NaCl in the bath, the mean V_{rev} of the ATP-activated current was $+1.29 \pm 2.01$ mV ($n = 10$). On reducing bath NaCl to 20 mM NaCl, the V_{rev} shifted to -24.4 ± 3.26 mV ($P < 0.001$), a mean shift of -25.6 ± 2.27 mV. This corresponded to a cation to anion permeability ratio of 15.5 ± 6.75 (Fig. 5a). Substitution of NaCl for RbCl or CsCl shifted the V_{rev} by $+5.65 \pm 1.35$ mV ($n = 9$, $P = 0.002$) and $+4.22 \pm 1.04$ mV ($n = 8$, $P = 0.003$) with RbCl and CsCl, respectively. LiCl was without effect on V_{rev} , $+1.37 \pm 2.14$ mV ($n = 8$, $P = 0.52$). These shifts corresponded to cation to Na⁺ selectivity ratios of 1.28 ± 0.07 , 1.22 ± 0.04 and 1.08 ± 0.10 , for Rb⁺, Cs⁺ and Li⁺, respectively. The V_{rev} of the ATP-activated current with Na⁺ in the pipette and NMDG⁺ in the bath was -29.4 ± 1.62 mV ($n = 7$) (Fig. 5b). This corresponded to an NMDG⁺:Na⁺ permeability ratio of 0.32 ± 0.02 . Addition of 5 mM Ca²⁺ to the extracellular solution shifted the V_{rev} by $+5.72 \pm 1.90$ mV ($n = 7$, $P = 0.017$) (Fig. 5c). This corresponded to a Ca²⁺:Na⁺ permeability ratio of 6.56 ± 2.98 . These data correspond to a selectivity sequence of Ca²⁺ > Rb⁺ = Cs⁺ > Na⁺ = Li⁺ > NMDG⁺.

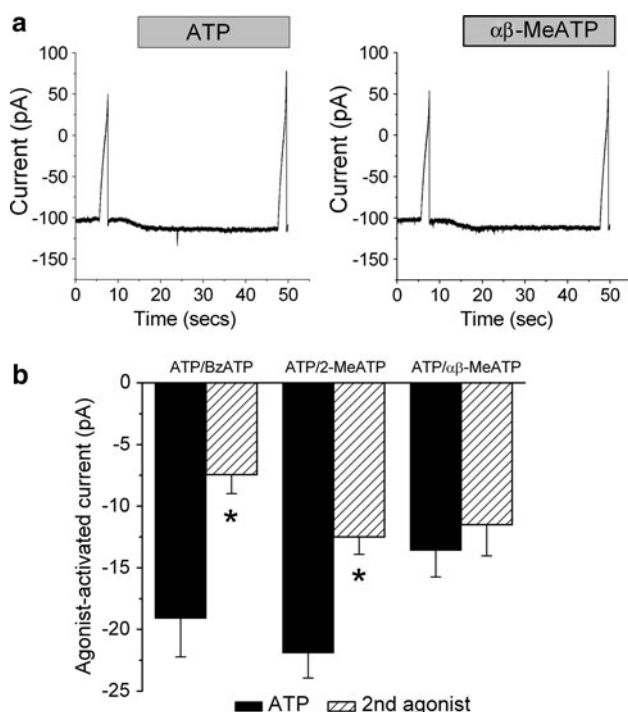


Fig. 2 Response to different P2X receptor agonists. Clamp potential was held at -100 mV. Under control conditions and in the presence of the agonist a ramp protocol was run between -100 and $+20$ mV. **a** Typical traces showing the effect of 500 μ M ATP (left) and $\alpha\beta$ -MeATP (right) in the same cell. **b** Mean agonist-activated current at -100 mV in paired cells exposed to ATP and a second agonist. * Significant difference from ATP

Effect of Extracellular Cations

Zn^{2+} increased the ATP-activated conductance (G_{ATP}) ($n = 10$, $P = 0.004$) (Fig. 6). The Zn^{2+} -mediated enhancement of G_{ATP} was reversible, with G_{ATP} returning to pre- Zn^{2+} levels on washout. In the Cu^{2+} experiments the effect of ATP was attenuated ($n = 7$, $P = 0.003$) (Fig. 6). The Cu^{2+} -mediated inhibition of G_{ATP} was reversible, with G_{ATP} returning to pre- Cu^{2+} levels on washout. With 0.5 mM Ca^{2+} in the bath G_{ATP} was 6.24 ± 0.74 μ S/cm² ($n = 16$). This was significantly reduced when bath Ca^{2+} was increased to 2 mM ($P = 0.03$) (Fig. 6). However, this reduction in G_{ATP} was not reversible. Acidification inhibited, while alkalization activated, G_{ATP} (mean data shown in Table 1).

Effect of P2X Activation on Whole-Cell K^+ Conductance

Whole-cell potential was clamped at -40 mV and then stepped to between $+20$ and -100 mV in -20 -mV steps. In control cells at time zero, quinidine inhibited a whole-cell current, I_{Quin} (Fig. 7a) ($P < 0.001$). The quinidine-sensitive

outward and inward conductances (GQ_{out} and GQ_{in}) were 7.65 ± 1.09 and 12.2 ± 1.56 μ S/cm², respectively ($n = 15$). The V_{rev} of I_{Quin} was -41.4 ± 3.84 mV (Fig. 7e), consistent with K^+ -selective currents. However, I_{Quin} decreased after 5 min (Fig. 7b). GQ_{out} and GQ_{in} were significantly reduced at 5.26 ± 0.60 and 7.38 ± 0.99 μ S/cm², respectively ($n = 15$, $P < 0.001$). The V_{rev} was unchanged, -39.16 ± 4.98 mV ($P = 0.46$) (Fig. 7e). In a separate population of test cells the responses to quinidine before and after the addition of ATP were determined. The initial GQ_{out} and GQ_{in} in the absence of ATP were 13.2 ± 2.18 and 23.6 ± 3.73 μ S/cm², respectively ($n = 17$). After 5-min exposure to ATP, these were unchanged, GQ_{out} 9.5 ± 1.47 and GQ_{in} 22.3 ± 3.50 μ S/cm² ($P > 0.12$) (Fig. 7f). The V_{rev} of I_{Quin} was -42.3 ± 4.07 and -41.8 ± 6.49 mV, initially and after 5-min exposure to ATP, respectively ($P = 0.93$) (Fig. 7f). Addition of ATP to the extracellular solution increased the outward and inward conductances by 8.26 ± 1.67 and 15.7 ± 2.20 μ S/cm², respectively, after 5 min ($n = 17$, $P < 0.0001$) (Fig. 7c). The V_{rev} of the ATP-activated current was -25.7 ± 4.11 mV (Fig. 7d). This was significantly depolarized in comparison to the V_{rev} of I_{Quin} ($P = 0.01$).

Role of P2X Receptors in Volume Regulation

Hypotonic shock elicited two responses, as described previously (Robson and Hunter 1994c). In 29 cells (42%), cell length increased to a peak, with recovery observed when cells were placed back in control Ringer. These were designated “non-RVD” cells (Fig. 8a, lower trace) and represent cells that require HCO_3^- for regulatory volume decrease (RVD) (Robson and Hunter 1994c). These cells were not considered further in the context of this study. In the remaining 40 cells (58%) cell length increased to a peak, followed by recovery toward the preshock level. These were designated “RVD” cells (Fig. 8a, upper trace). The initial length of the RVD cells was 21.2 ± 0.34 μ m ($n = 40$). Hypotonic shock increased this by 0.80 ± 0.04 μ m ($P < 0.0001$), followed by recovery. Steady-state length was 0.13 ± 0.05 μ m above the preshock level at steady state after volume regulation.

A possible role for extracellular ATP in RVD was examined by exposing cells to ATP/ADP apyrase. Neither the proportion of cells undergoing RVD (64%, $n = 7$, Fisher’s exact probability test) nor the initial length of the cells (22.4 ± 0.76 μ m, $n = 7$, unpaired Student’s *t*-test) was different from control RVD cells. However, ATP/ADP apyrase inhibited RVD in comparison to control cells (unpaired Student’s *t*-test). Figure 8b shows the increase to peak and steady-state length after RVD relative to initial length for apyrase and day-matched controls (control 1).

In a second series of experiments, two types of response were observed in control cells, as described previously,

Fig. 3 Effect of antagonists on the 500 μM ATP-activated current. Cells were held at -100 mV and then exposed to 500 μM ATP in the absence and then the presence of the antagonist. This was followed by a second control exposure to ATP after washout of the antagonist. **a-c** Typical whole-cell traces at -100 mV from three different cells in the absence and presence of 100 μM suramin, 30 nM TNP-ATP or 10 μM PPADS, respectively. *Arrows* indicate when 500 μM ATP was added to the bath. **d** Mean ATP-activated currents. * Significant reduction compared to the first response to ATP. *Black bars* indicate ATP, *hatched bars* indicate ATP plus antagonist and *gray bars* indicate ATP

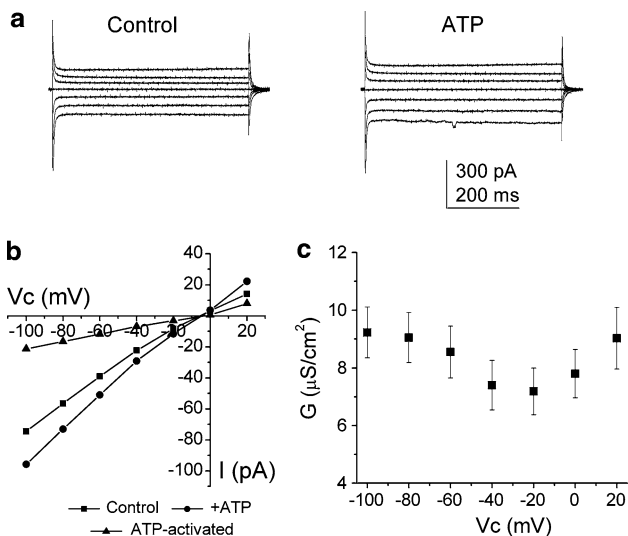
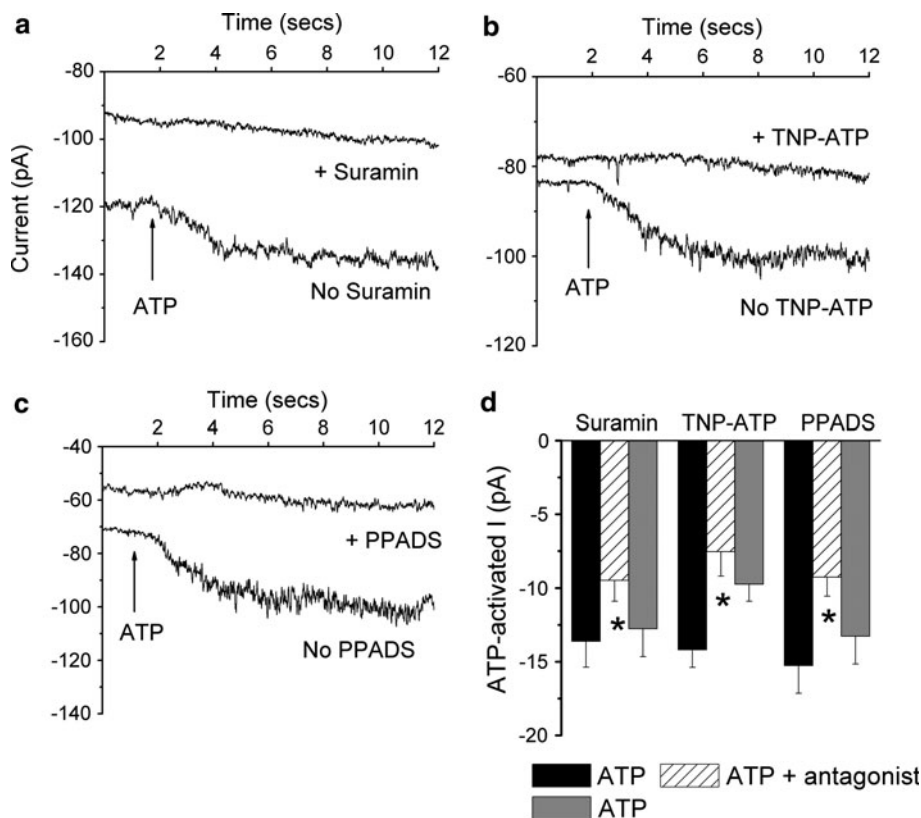


Fig. 4 Effect of ATP on whole-cell currents. Cells were clamped at -40 mV and then potential stepped to between $+20$ and -100 mV in -20-mV steps. **a** Typical traces obtained from the same cell in the absence (*left*) and presence (*right*) of 2 mM ATP. **b** *I-V* curves generated from the traces shown in **a**. *Vc* is the command voltage. **c** Mean point conductance of the ATP-activated currents ($n = 14$)

RVD (47%) and non-RVD cells (53%). In RVD cells initial length was $22.2 \pm 0.46\ \mu\text{m}$ ($n = 22$). On exposure to a hypotonic shock, length increased by $0.66 \pm 0.04\ \mu\text{m}$, to a

peak of $22.9 \pm 0.48\ \mu\text{m}$ ($P < 0.001$), followed by RVD. Length was $0.06 \pm 0.08\ \mu\text{m}$ above the preshock level at steady state after volume regulation. Under the experimental conditions the proportions of cells undergoing RVD were 75% (21) for 100 μM suramin, 63% (12) for 10 μM PPADS and 61% (14) for 30 nM TNP-ATP. The number of RVD cells was significantly increased for suramin but not PPADS or TNP-ATP (Fisher's exact probability test). The initial lengths of RVD cells were 22.9 ± 0.47 ($n = 21$), 21.7 ± 1.06 and 22.2 ± 0.54 ($n = 14$) μm in the presence of suramin, PPADS and TNP-ATP, respectively. Figure 8b shows the increase to peak and steady-state length after RVD relative to initial length for all experimental conditions. Suramin, TNP-ATP and PPADS all inhibited RVD in comparison to the control circumstance, $F_{3,65} = 5.57$ (all tested using ANOVA).

Discussion

These data provide evidence for the functional expression of a P2X-like current in frog renal proximal tubule cells, P2X_f. P2X_f was cation-selective, did not discriminate well between monovalent cations, had a poor permeability to NMDG and was around six times more permeable for Ca²⁺ over Na⁺. It was activated by a variety of P2X agonists,

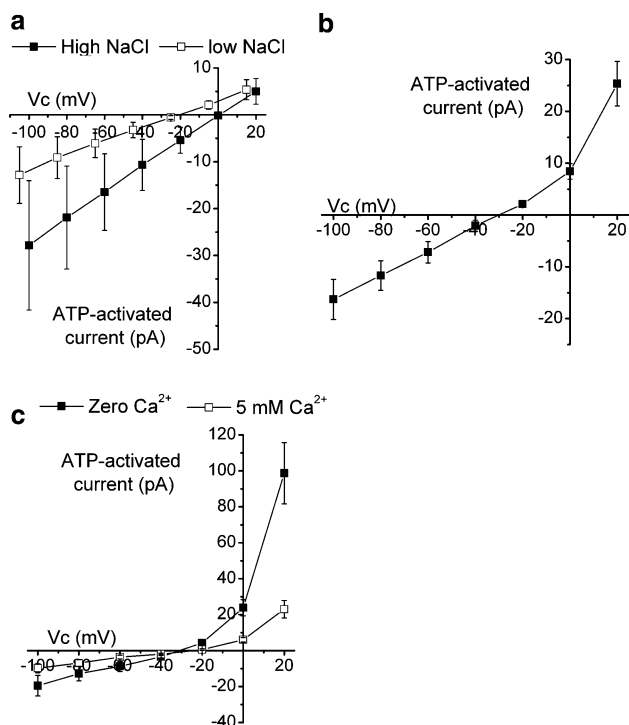


Fig. 5 Selectivity of ATP-activated current. Cells were clamped at -40 mV and then potential stepped to between $+20$ and -100 mV in -20 -mV steps. V_c is the command voltage. **a** Mean currents recorded in high (filled square) or low (open square) NaCl in paired cells ($n = 10$). Note the shift along the x axis of the low-NaCl data due to junction potential correction. **b** Mean ATP-activated current recorded with NaCl in the pipette and NMDG-Cl in the bath ($n = 7$). **c** Mean ATP-activated currents recorded with bath NMDG-Cl in the absence of Ca^{2+} (filled square) or in the presence of 5 mM Ca^{2+} (open square) in paired cells ($n = 7$)

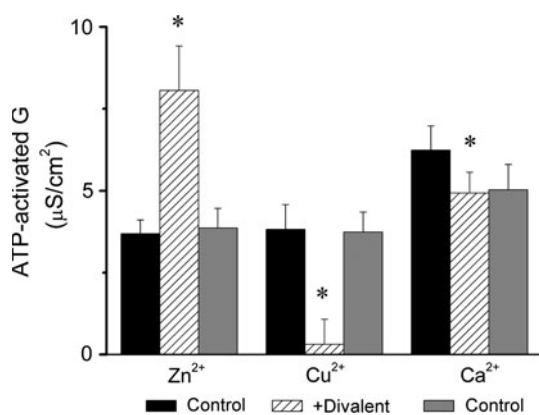


Fig. 6 Effect of divalent cations on G_{ATP} . Cells were clamped at -40 mV and then potential stepped to between $+20$ and -100 mV in -20 -mV steps. The graph shows the effect of Zn^{2+} , Cu^{2+} and Ca^{2+} . * Significantly different response from ATP in the presence of the divalent cation. Black bars indicate ATP, hatched bars indicate ATP plus divalent cation and gray bars indicate ATP

including ATP, BzATP, 2MeSATP and $\alpha\beta$ -MeATP, and was sensitive to some P2X antagonists. P2X_f also demonstrated inward rectification, a property shared by some P2X receptors (Evans et al. 1996). These properties are consistent with P2X_f being attributable to a P2X receptor. However, one difference from P2X receptors was that it was activated only by high concentrations of extracellular ATP, 100 μM to 10 mM. In contrast, most of the cloned P2X receptors require only micromolar levels of ATP (Torres et al. 1998; Valera et al. 1994; Virginio et al. 1998b), although P2X_7 is activated by high levels of ATP (Rassendren et al. 1997). Consistent with LLC-PK₁ cells (Filipovic et al. 1998), activation of P2X_f was observed with 100 μM ATP, although maximal activation required millimolar levels. The reason for this difference is not clear, but it may reflect the fact that the current study involves an amphibian P2X receptor. Certainly, a study on cloned *Xenopus* P2X_4 also used high concentrations of ATP (Juranka et al. 2001). In addition, frog P2X receptors in aorta require similarly high concentrations of agonists for activation (Knight and Burnstock 1996), with maximal activation not observed with 3 mM ATP. Alternatively, it is known that the proximal tubule membrane contains ecto-ATPases that break down ATP (Huang et al. 2006). Therefore, another explanation could be that the ATP in the extracellular solution next to the membrane was at a lower concentration than the bulk solution. The Hill coefficients for ATP and BzATP were similar to other studies (Jiang et al. 2003), with the larger coefficient for ATP suggesting greater cooperativity of binding compared to BzATP. In terms of the in vivo luminal ATP concentration in the proximal tubule, there is a general lack of information, with one study suggesting a maximal concentration of around 275 nmol/l in rats (Vekaria et al. 2006). This concentration would not be sufficient to activate P2X_f and, indeed, is also on the low side for activation of mammalian P2X receptors. However, this concentration reflects the mean in the tubular fluid, and one suggestion is that ATP concentrations closer to the membrane could be much higher. If this is the case, then it is possible that sufficiently high concentrations are reached for activation of both mammalian and amphibian P2X receptors. In addition, as discussed later, it is clear that activation of P2X_f can impact on the physiological function of the frog renal proximal tubule.

What are the properties of P2X_f , and how do these compare to the different cloned P2X receptors? Two amphibian cloned receptors have been identified and show 67% homology with rat P2X_4 and P2X_5 (Jensik et al. 2001; Juranka et al. 2001). P2X_f shows some similarities to these cloned receptors and to some heteromeric mammalian receptors (Table 2). The two amphibian P2X receptors can be activated by levels of ATP, below 100 μM . This is

Table 1 Effect of extracellular pH on the ATP-activated current

I_{ATP} -100 mV (pA)	(n = 9)	I_{ATP} -100 mV (pA)	(n = 9)
pH 7.4	-21.4 ± 2.94	pH 7.4	-19.0 ± 4.03
pH 6.3	-12.2 ± 2.73*	pH 8.3	-30.2 ± 6.45*
pH 7.4	-20.1 ± 4.23	pH 7.4	-24.8 ± 5.01

* Significant difference from pH 7.4 ($P < 0.029$)

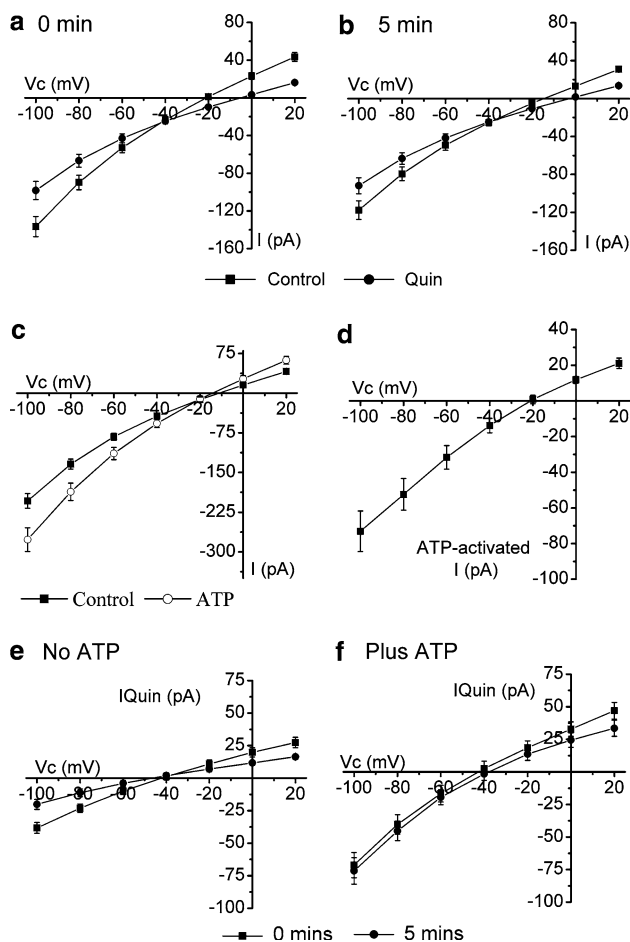


Fig. 7 Effect of quinidine and ATP on whole-cell currents. Cells were clamped at -40 mV and then potential stepped to between +20 and -100 mV in -20-mV steps. V_c is the command voltage. **a, b** Mean whole-cell currents recorded in the absence of ATP initially on achieving the whole-cell configuration (**a**) and after 5 min (**b**). In both figures filled square indicates currents recorded in the absence of quinidine, while filled circle indicates currents recorded in the presence of quinidine ($n = 15$ for all). **c** Effect of addition of extracellular ATP on mean whole-cell current. Filled square, currents recorded in the absence of ATP; open circle, currents recorded after 5-min exposure to ATP ($n = 17$). **d** Mean ATP-activated current. **e, f** Quinidine-sensitive currents in the absence of ATP (**e**) or presence of ATP (**f**). In both graphs filled square indicates currents recorded initially after achieving the whole-cell configuration, while filled circle indicates currents recorded 5 min later

different from $P2X_f$, where 100 μ M ATP was needed for activation. However, as neither study performed a dose response to ATP, a definitive comparison cannot be made.

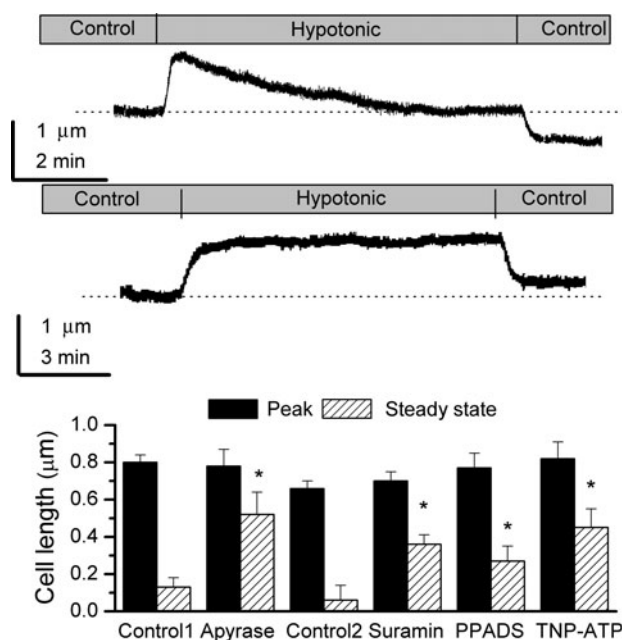


Fig. 8 Effect of P2X antagonists on RVD. **a** Typical cell length traces showing an RVD cell (upper) and a non-RVD cell (lower). Dotted lines represent the preshock level. **b** Effect of apyrase, suramin, PPADS and TNP-ATP on RVD. Data are expressed relative to control length, with 0 representing the initial preshock level. * Significant difference from the control circumstance. *Control1* shows day-matched data for the apyrase experiments; *Control2* shows the day-matched data for the suramin, PPADS and TNP-ATP experiments

In divalent free conditions neither cloned P2X receptor demonstrated rapid desensitization on exposure to ATP, similar to $P2X_f$. However, although desensitization was not observed with $P2X_f$ when bath Ca^{2+} was low, an apparent desensitization was observed when the cells were exposed to 2 mM Ca^{2+} . Under this circumstance poor recovery of the response to ATP was observed on washout of Ca^{2+} from the bath. This decrease in the response to ATP during several exposures was not observed with low Ca^{2+} and is consistent with the Ca^{2+} -dependent desensitization observed in amphibian P2X receptors. The agonist potency sequence of $P2X_f$, $ATP = \alpha\beta meATP > BzATP = 2MeSATP$, is similar to the frog aorta P2X receptor (Knight and Burnstock 1996). This suggests that $P2X_f$ is not attributable to $P2X_7$ (Rassendren et al. 1997), although variations in the response to BzATP are seen at $P2X_7$ receptors from different species (Fonfria et al. 2008). It is therefore not

Table 2 Comparison of the properties of P2X_f with cloned amphibian and mammalian heteromeric receptors

	Amphibian P2X ₄	Amphibian P2X ₅	P2X _{2/3}	P2X _{1/4}	P2X _{1/5}	P2X _{4/6}	References
Agonist potency		X	◆	◆	◆	X	Jensik et al. (2001)
Suramin		>	>	>	>	◆ 40%	Juranka et al. (2001) Le et al. (1998)
PPADS	◆	>	>	>	>	◆	Le et al. (1999)
TNP-ATP			>	>	>		Liu et al. (2001)
Zn ²⁺			◆			◆	Nicke et al. (2005)
Ca ²⁺		◆	◆		X		Stoop et al. (1997)
pH			X		◆	◆	Virginio et al. (1998a) Wildman et al. (1998)

Percentages indicate percentage inhibition values close to those observed for P2X_f

◆, Similarity to P2X_f; X, difference; >, higher sensitivity; blank, not known

possible to absolutely rule out P2X_f being attributable to a P2X₇-like receptor, although the P2X₇ antagonists KN-62 and BBG were without effect on P2X_f (Humphreys et al. 1998; Jiang et al. 2000). P2X_f was sensitive to 100 μM suramin, 10 μM PPADS and 30 nM TNP-ATP (30%, 44% and 34% inhibition, respectively). This sensitivity to PPADS is similar to amphibian P2X₄ (50% inhibition) (Juranka et al. 2001) and P2X_{4/6} (40% inhibition) (Le et al. 1998), although it is different from the bullfrog P2X receptor, which is completely blocked by both 100 μM suramin and PPADS (Jensik et al. 2001). The sensitivity to PPADS was similar to amphibian P2X₄ and P2X_{4/6}. TNP-ATP is a potent inhibitor of P2X_{1/5}, P2X_{1/4} and P2X_{2/3} (Le et al. 1999; Nicke et al. 2005; Virginio et al. 1998b), with ~80% inhibition observed with 30 nM. This is higher than the sensitivity of P2X_f. Like P2X_f, the bullfrog cloned receptor also shows inhibition by Ca²⁺ (Jensik et al. 2001). P2X_f was potentiated by ivermectin, which is known to enhance activation of P2X₄ (Priel and Silberberg 2004) and was also potentiated by extracellular Zn²⁺ and inhibited by extracellular Cu²⁺ and H⁺.

Overall, P2X_f would appear to share greatest similarity with P2X_{2/3} or P2X_{4/6}, although its properties are not entirely consistent with these heteromeric P2X receptors. Given the fact that previous expression studies have shown the presence of P2X₁, P2X₄, P2X₅ and P2X₆ in the renal proximal tubule, it is likely that P2X_f represents a P2X_{4/6}-like P2X receptor.

P2X_f is clearly a native proximal tubule P2X-like receptor, and on activation it would be expected to lead to an influx of Ca²⁺ into the cell. However, the magnitude of the P2X_f-mediated currents was small. In comparison to P2X receptor expression studies this is to be expected as in those studies the magnitude of the P2X currents reflects the fact that the channels have been overexpressed. Looking at studies of native P2X receptor currents provides a variable

picture in terms of current magnitude. In LLC-PK₁ cells, currents were on the order of a few hundred picoamperes (Filipovic et al. 1998). In contrast, studies in neurons, airway ciliated cells and Leydig cells show small P2X-mediated currents, similar to P2X_f (Chaves et al. 2006; Ma et al. 2006; Mori et al. 2001). It is clear, therefore, that physiologically relevant P2X native currents can be small. What is the physiological role of P2X_f in the renal proximal tubule? The data presented in the current study suggest that P2X_f plays an important role in volume regulation and K⁺ channel activation.

Frog proximal tubule cells have the ability to regulate their volume in response to cell swelling, RVD (Robson and Hunter 1994c). Such volume regulation plays an important role in diverse cellular process such as cell growth and proliferation, osmoregulation and cellular metabolism. There is a clear role for P2 activation in regulating proximal tubule cell proliferation (Lee and Han 2006), while a role for P2 receptor activation in RVD in hepatocytes has been proposed (Wang et al. 1996). This suggests that P2 receptors and volume regulation may be important for normal cell function. Previous work in frog proximal cells has demonstrated a role for K⁺ and Cl⁻ channels in RVD (Robson and Hunter 1994c, 2005). Whole-cell patch-clamp experiments have identified barium-sensitive K⁺ and DIDS-sensitive Cl⁻ currents that are volume sensitive (Robson and Hunter 1994a, 2005). Unpublished studies demonstrate that quinidine-sensitive K⁺ currents are also volume-sensitive, with quinidine-sensitive conductance reduced in the presence of a hypertonic bath solution (19.2 ± 4.38 vs. 7.31 ± 1.46 μS/cm² in the presence of control and hypertonic solutions, respectively, n = 12 for each group). For the Cl⁻ channels volume activation is mediated by protein kinase C (PKC) (Robson and Hunter 1994a). The mechanism underlying volume regulation of the K⁺ currents has not been

elucidated but does not appear to involve PKC. In addition, RVD is inhibited in the absence of extracellular Ca^{2+} , consistent with a Ca^{2+} influx pathway playing a critical role (Robson and Hunter 1994c). The specific mechanism by which Ca^{2+} enters the renal proximal is unknown; however, a number of candidates have been proposed. One of these is stretch-activated, Ca^{2+} -permeable cation channels (SACs), as the SAC inhibitor gadolinium (Gd^{3+}) blocks RVD (Robson and Hunter 1994c) and two Gd^{3+} and volume-sensitive cation conductances have been identified in these cells (Robson and Hunter 1994b). However, subsequent work has indicated that the volume-sensitive Cl^- channels are also inhibited by Gd^{3+} (Robson and Hunter 1994a). Therefore, the effect of Gd^{3+} on RVD could simply reflect inhibition of Cl^- efflux rather than Ca^{2+} influx. A second possibility is that, on cell swelling, there is release of ATP from the cells, with ATP subsequently leading to Ca^{2+} influx via the activation of P2X receptors. This is supported by the current study, which demonstrated that in the presence of apyrase RVD was inhibited, suggesting that the release and presence of ATP are important in initiating volume regulation. RVD was also inhibited by the P2X antagonists suramin, TNP-ATP and PPADS. This supports a role for P2X receptor activation in RVD. The degree of inhibition of RVD was similar to that observed with P2X_f .

These data support a role for P2X_f activation in RVD and suggest that it may provide the Ca^{2+} influx pathway. This influx of Ca^{2+} would be expected to activate downstream efflux pathways, such as the K^+ and Cl^- channels described earlier. A clear link exists to activation of the Cl^- channels as this is PKC-mediated. For the activation of K^+ channels a rise in intracellular Ca^{2+} could directly activate channels or work indirectly via Ca^{2+} -dependent signalling systems. The whole-cell K^+ current data described here suggest that P2X_f plays an important role in the activation of quinidine-sensitive K^+ channels previously observed in the cells. In the absence of extracellular ATP, whole-cell quinidine-sensitive K^+ currents decreased over 5 min. However, in the presence of extracellular ATP this rundown was absent, indicating that extracellular ATP was able to inhibit the rundown process. The V_{rev} of the quinidine-sensitive currents, around -40 mV, suggests that the K^+ channel regulated by extracellular ATP was a previously identified K^+ conductance (Robson and Hunter 1997). The total ATP activated current demonstrated inward rectification, similar to P2X_f . Interestingly, the V_{rev} of the total ATP-activated current was more positive than the K^+ currents but more negative than the V_{rev} for P2X_f . This suggests that at least part of the ATP-activated current may reflect activation of K^+ -selective channels. Activation of K^+ channels via P2X receptor activation has been observed in rat osteoclasts and toad gastric smooth muscle

cells (Weidema et al. 1997; Zou et al. 2001), with P2Y_2 -mediated inhibition of K^+ channels in the mouse cortical collecting duct (Lu et al. 2000).

In conclusion, the current study provides the first report of a native P2X receptor in renal proximal tubule cells. The receptor, P2X_f , was cation-selective, did not discriminate between cations and was Ca^{2+} -permeable. P2X_f was activated by the purines $\text{ATP} = \alpha\beta\text{meATP} > \text{BzATP} = 2\text{MeSATP}$, did not demonstrate fast desensitization and was inhibited by suramin, PPADS and TNP-ATP. P2X_f -mediated currents were enhanced in the presence of Zn^{2+} or ivermectin and inhibited in the presence of Cu^{2+} or on acidification. These properties are consistent with P2X_f being attributable to a P2X receptor and suggest that P2X_f may be attributable to a heteromeric receptor, with $\text{P2X}_{4/6}$ a possible candidate. The evidence presented suggests that activation of P2X_f plays a role in the regulation of cell volume and K^+ channels in frog renal proximal tubule cells.

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