P2X receptors trigger intracellular alkalization in isolated perfused mouse medullary thick ascending limb

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Abstract

Aims: Extracellular ATP is an important regulator of renal tubular transport. Recently, we found that basolateral ATP markedly inhibits Na⁺ and Cl⁻ absorption in mouse medullary thick ascending limb (mTAL) via a P2X receptor. The underlying mechanism that mediates this ATP-dependent transport inhibition in mTAL is, however, unclear. The renal outer medullary K⁺ channel (ROMK) is sensitive to intracellular pH where a reduction leads to closing of ROMK. We speculated that P2X receptor stimulation in the TAL could lead to changes in pHᵢ, leading to a reduction in NaCl transport.

Methods: To test this hypothesis, we measured pHᵢ in single perfused mouse mTALs using the fluorescent ratiometric dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester.

Results: Interestingly, basolateral ATP (100 μM) caused a prominent, reversible intracellular alkalization of mTAL, with an average pHᵢ increase of 0.14 ± 0.02 (n = 14). This was completely abolished by the P2X receptor antagonist periodate-oxidized ATP (50 μM). The P2X receptor-mediated intracellular alkalization required the activity of the apical Na⁺/H⁺ exchanger (NHE3). Typically, Gq-coupled receptors cause a significant acidification of tubular epithelial cells, which was confirmed in this study, by P2Y₂ and Ca²⁺ sensing receptor stimulation.

Conclusion: This study reports that stimulation of basolateral P2X receptors causes a substantial intracellular alkalization in the isolated perfused mouse mTAL. This intracellular alkalization is mediated through an increased apical NHE3 activity, similar to what we previously observed when tubular transport is inhibited with furosemide. This increased NHE3 activity causes H⁺ secretion in the mTAL and provides further support that the TAL is a site of urinary acidification.

Keywords intracellular pH, loop of Henle, Na⁺/H⁺ exchanger 3, P2 receptors, purinergic.

The thick ascending limb of Henle’s loop (TAL) is essential for overall body water and salt homeostasis. The TAL reabsorbs 20–30% of the filtered NaCl load and creates the osmotic gradient in the renal medulla, thereby facilitating H₂O reabsorption in the collecting duct. Na⁺ uptake in the TAL is mediated by the Na⁺/K⁺ Cl⁻ cotransporter NKCC2 (Greger 1985). Na⁺ leaves the TAL through the basolateral Na⁺/K⁺ ATPase (Greger 1985), and Cl⁻ exits via the basolateral Cl⁻ channel ClCₖb (Greger 1985). K⁺ is partially recycled into the lumen by the renal outer medullary K⁺ channel (ROMK). Inhibition of ROMK with 3 mM Ba²⁺ results in a complete block of Na⁺ and Cl⁻ reabsorption in TAL (Greger & Schlatter 1983). ROMK...
has been shown to be sensitive to intracellular pH (pHi), where a minor cytosolic acidification results in closing of the channel (Bleich et al. 1990, Choe et al. 1995, Lehrmann et al. 1990, Bailey 2004, Rieg et al. 2007, Pochynyuk et al. 2008). The TAL has been shown to express functional apical P2Y2 receptors and basolateral P2Y2 and P2X receptors. Stimulation of the P2 receptors of either type results in rises in [Ca\textsuperscript{2+}], (Jensen et al. 2007, Geyti et al. 2008), and extracellular ATP is known to reduce O\textsubscript{2} consumption in TAL suspensions, which is the likely result of Na\textsuperscript{+} and Cl\textsuperscript{−} transport inhibition (Silva & Garvin 2009). Indeed, transport measurements in isolated perfused medullary TAL (mTAL) show that basolateral ATP causes an inhibition of Na\textsuperscript{+} and Cl\textsuperscript{−} transport that is mediated through multiple P2X receptors, including the P2X\textsubscript{4} receptor (Marques et al. 2012). The mechanism for this transport inhibition is not yet established. In this study, we wanted to investigate whether extracellular ATP induces changes in pHi in isolated perfused mouse mTAL. It was speculated that stimulation of P2X receptors could cause an intracellular acidification, which potentially could trigger transport inhibition. In several studies that use agonists to elevate [Ca\textsuperscript{2+}], it has been established that a rise in [Ca\textsuperscript{2+}] is associated with intracellular acidification (Berk et al. 1987, Sage et al. 1990). This is also the case for P2X receptors, where stimulation associates with an intracellular acidification (Henriksen & Novak 2003). Thus, P2X\textsubscript{4} receptor stimulation could potentially result in a decrease in cytosolic pHi, thereby closing ROMK to cause an inhibition of Na\textsuperscript{+} and Cl\textsuperscript{−} transport in the TAL.

This hypothesis was proven wrong and, surprisingly, basolateral ATP caused a significant, sustained and reversible intracellular alkalinization through P2X4 receptor stimulation in perfused mouse mTALs. The ATP-induced increase in pHi is mediated by the apical Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 (NHE3) and can only be observed in an actively transporting tubule. Our data imply that ATP triggers NHE3-dependent H\textsuperscript{+} secretion through an inhibition of tubular transport. The results also reflect a potential role of the TAL in urinary acid secretion.

**Materials and methods**

**Tubule perfusion**

All mouse handling of animals complied with Danish animal welfare regulations. Animals had free access to standard rodent diet and tap water. Experiments were performed on 4- to 6-week-old mice with a mixed genetic background (B6D2/SV129). Mice were sacrificed by cervical dislocation, the kidneys collected, placed in ice-cold control solution containing (in mM) 145 NaCl, 0.4 KH\textsubscript{2}PO\textsubscript{4}, 1.6 K\textsubscript{2}HPO\textsubscript{4}, 5 d-glucose, 1 MgCl\textsubscript{2}, 1.3 Ca-gluconate and 5 N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES) and were subsequently sliced. The slices were placed in a dissection chamber with cold (4 °C) control solution. mTALs were isolated from the inner stripe of the outer medulla (ISOM) with ultralfine forceps. The dissected mTALs were transferred to a perfusion chamber mounted on an inverted microscope (Axiovert 100 TV; Zeiss, Jena, Germany) and perfused with a concentric pipette system as described previously (Greger & Hampel 1981). TALs were stabilized on the bath bottom with a holding pipette. Tubules were bathed and perfused with control solution from one side with the tubule outflow left open. All experiments were performed at 37 °C, and agonist and antagonist solutions were prepared fresh.

**Fluorescence recording**

The set-up for fluorescence microscopy consisted of an inverted microscope with a 63× C-Apochromat 1.2 water (Zeiss) objective, a VisiChrome polychromator system (Visitron, Puchheim, Germany) and a digital CCD camera (Spot pursuit 1.4 monochrome; Diagnostics Instruments, Sterling Heights, MI, USA). Images were acquired, and data analysed with standard software (VISIVIEW; Visitron). Intracellular pH was measured with the ratiometric fluorescent dye 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF AM; Invitrogen, Carlsbad, CA, USA). Tubules were incubated with 5 μM basolateral BCECF AM in control solution for 20 min at RT during continuous perfusion with control solution, followed by a 5-min washout period. The pHi was measured as the emission ratio at 490/436 nm excitation during 5-s intervals. To reduce photo damage to the tissue, the excitation speed was 50 ms at 436 nm and 25 ms at 490 nm. A 500-nm beam splitter and a 520-560 band pass were used. Experimental manipulations were carried out after a stable fluorescence signal was
achieved, and fluorescence of the entire tubule was recorded for analysis. ATP, oxidized ATP (oATP) (Sigma-Aldrich) and #4167 (kindly provided by Sanofi Aventis, Germany) were freshly dissolved in H2O prior experiments. Furosemide was dissolved in DMSO to 0.1M stock solution and dissolved further in H2O prior to each experiment.

Calibration of the BCECF signal was performed using the high K+ and nigericin method (Thomas et al. 1979) in a paired fashion. The calibration solution was used previously in similar experiments (Watts & Good 1994, Odgaard et al. 2004) and contained (in mM) 95 KCl, 15 NaCl, 0.4 NaH2PO4, 1.6 Na2HPO4, 5 glucose, 1 MgCl2, 1.3 Ca-gluconate, 25 HEPES and 20 N-Methyl-D-glucamine, supplemented with 2 lM nigericin. The solution was set at pH 6.5, 7.4 and 7.8.

Statistics

All data are presented as mean ± SEM in all series, n indicates the number of tubules used. For each series, no more than two tubules were used from a single mouse. Data were tested for normality with the Kolmogorov–Smirnov test. Differences between experimental conditions were analysed using the paired or unpaired Student’s t-test or ANOVA where necessary. In all cases, P < 0.05 was considered significant.

Results

Basolateral ATP induces an alkalization in mTAL cells

Figure 1a shows a typical pHi recording of a perfused mTAL. Under resting conditions, the pHi of this tubule was 7.28. The average pHi of the summarized data from all perfused mTALs was 7.31 ± 0.05 (n = 14, Fig. 1b). Application of basolateral ATP (100 µM) caused a reversible intracellular alkalization in perfused mTAL, which was sustained during the 2-min exposure to ATP and then returned to the basal pH after a washout period of 2–5 min. The average pHi during ATP exposure was 7.45 ± 0.06, and after 5-min washout, the pH recovered to 7.31 ± 0.05 (Fig. 1b, P < 0.0001). These data reflect that ATP causes an intracellular alkalization with a magnitude of 0.14 ± 0.02 pH units. Figure 2 shows the concentration–response curve of ATP in a range from 100 nM to 500 µM. The estimated EC50 was 6.74 ± 1.67 µM. Interestingly, low concentrations of ATP (1 µM) resulted in a decrease in pH, suggesting that basolateral ATP has a dual effect on intracellular pH in mouse mTAL.

P2Y2 receptor stimulation causes an intracellular acidification

Our group previously demonstrated that of the P2Y receptor family, only P2Y2 and P2Y6 are expressed in murine mTAL (Marques et al. 2012). To investigate the role of P2Y receptors in the ATP-induced alkalization, we used the potent P2Y2 receptor agonist UTP that does not activate P2X receptors. Figure 3a shows a typical experiment of a perfused mTAL exposed to basolateral UTP (100 µM), causing a small but significant acidification (ΔpHi 0.04 ± 0.01, n = 7, P < 0.001). UDP, a specific P2Y6 receptor agonist, did not cause any changes in pH (results not shown). The magnitude of the UTP-induced acidification was similar to that seen when 1-µM ATP was applied to the basolateral side (ΔpHi 0.05 ± 0.01, n = 6, Fig. 3b), which is sufficient to stimulate the P2Y2 receptor (Abbracchio et al. 2006). Stimulation of the Ca2+ sensing receptors (CaSR) with increased extracellular [Ca2+] (5 mM) also acidified perfused mTALs. These

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Figure 1 Basolateral ATP causes an intracellular alkalization in perfused medullary thick ascending limb (mTAL). (a) Representative trace of a perfused mTAL loaded with 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF AM). ATP (100 µM) is applied to the bath for 2 min. (b) Summarized data of the experiments with 100 µM basolateral ATP, n = 14. *Indicates statistical significance (P < 0.001).
findings indicate that stimulation of Gq-protein-coupled receptors such as P2Y2 and CaSR in the TAL causes a small intracellular acidification.

**The ATP-induced alkalization is mediated through P2X receptors**

We previously demonstrated that P2X1, P2X4 and P2X5 receptors are expressed in TAL of mice (Marques et al. 2012). To confirm that the ATP-induced alkalization is mediated by P2X receptor stimulation, we used the unspecific irreversible P2X receptor antagonist oATP. Figure 4a shows an original trace of a time control experiment, for two consecutive applications of basolateral ATP to a perfused mTAL separated by 12-min washout. It is clear from both Figure 4a and the summarized data in Figure 4b that ATP induced an alkalization of comparable size in both cases. When, however, ATP was applied after the irreversible P2X receptor antagonist oATP (50 µM), the ATP-induced alkalization was completely abolished (Fig. 4c). Instead, an acidification was observed, congruent with a residual P2Y2 receptor stimulation (Fig. 4d). Thus, the ATP-induced alkalization is mediated through basolateral P2X receptors.

**ATP-induced alkalization is mediated through activation of apical NHE3**

A recent study from our group has established that furosemide causes an intracellular alkalization in the mTAL through increased apical NHE3-mediated H⁺ secretion (de Bruijn et al. 2013). To investigate whether the ATP-induced intracellular alkalization occurs by the same mechanism, basolateral ATP was tested in the presence of the specific NHE3 blocker #4167 (Reuter et al. 2008). Figure 5а shows that luminal #4167 (1 µM) caused a significant intracellular acidification (ΔpH = −0.35 ± 0.02, n = 8, Fig. 5b). During this NHE3 inhibition, the ATP-induced alkalization was completely abolished in five of eight experiments, whereas in three experiments, the alkalization was strongly attenuated. These data indicate that apical NHE3 activity is required for the ATP-induced alkalization.

**ATP-induced alkalization is abolished in the presence of furosemide**

Furosemide and ATP both cause significant intracellular alkalizations in mTAL that require NHE3 activity (this study and de Bruijn et al. 2013), and they are
known to reduce tubular transport in this segment (Greger 1985, Marques et al. 2012). It was therefore interesting to study whether the pHi effects of the two substances are additive. Figure 6a illustrates that furosemide causes a marked intracellular alkalization from pH 7.25 ± 0.08 to pH 7.60 ± 0.02 (n = 6, Fig. 6b). When basolateral ATP (100 µM) was added during the continuous presence of luminal furosemide, pH i remained unchanged. These results do not support an additive effect of these two alkalizing stimuli and thus are consistent with the notion of a common underlying cause.

**Discussion**

In this study, we describe the surprising finding that basolateral P2X receptor stimulation triggers a marked alkalization in the isolated perfused mouse mTAL. It is well established that GPCRs and ligand-gated ion channels in a variety of tissues trigger a significant intracellular acidification (Berk et al. 1987, Sage et al. 1990, Henriksen & Novak 2003). We were therefore curious to define the mechanism for the pronounced ATP-triggered alkalization. As a first step, we confirmed that P2Y2 receptor and CaSR stimulation triggered the expected acidification in the mTAL. We further showed that the ATP-stimulated alkalization occurred via basolateral P2X receptors. As the experiments were conducted in HEPES buffer, the ATP-stimulated alkalization must reflect the removal of protons from the cytosol. Finally, we demonstrated that the P2X receptor-mediated alkalization was completely inhibited by blocking the apical NHE3. In summary, our results are consistent with an ATP-activated H+ secretion into the tubular lumen via the Na+-dependent H+ exchanger NHE3 in mTAL from mice.

Clues to a mechanism of how ATP activates NHE3 can be found in a parallel report from our group (de Bruijn et al. 2013). In this work, we demonstrated that furosemide causes a prominent intracellular alkalization, which results from a sudden drop in [Na+]i that apparently leads to an increased activity of the apical NHE3 by a more favourable chemical Na+ gradient. The marked drop of [Na+]i is best explained by

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acute inhibition of Na\(^+\) influx via the apical NKCC2 cotransporter during continuous basolateral Na\(^+\) efflux via the Na\(^+\)/K\(^+\) ATPase. Moreover, it was shown that furosemide stimulated H\(^+\) secretion into the mTAL lumen by directly measuring luminal pH. One main conclusion of this parallel work was that transport inhibition with loop diuretics triggers a marked H\(^+\) secretion and therefore urinary acidification.

In this study, we identify that basolateral P2X receptor stimulation leads to the activation of the apical NHE3. However, the cellular mechanism could not be resolved. It is well established that NHEs are activated by cell shrinkage and thus involved in regulatory volume increase (RVI, Hoffmann et al. 2009). However, NHE3 has been demonstrated to be activated by cell swelling rather than shrinkage in rat TAL (Watts & Good 1999, Good et al. 2000). Activation of the P2X receptors results in the influx of cations and, therefore, could result in cell swelling. Preliminary data confirm that ATP causes cell volume increases in perfused mTAL and show that hyposmolality result in an intracellular alkalization (unpublished data). It is thus likely that the observed intracellular alkalization caused by P2X receptor stimulation is a consequence of cell swelling-induced NHE3 activation. However, Watts and Good have shown that this swelling-induced activation of NHE3 still takes place in the presence of furosemide (Watts & Good 1999). Our data with furosemide and ATP show that NHE3 can apparently not be further stimulated by P2X receptor activation (Fig. 6). More detailed studies on the activation of NHE3 in both cases are required to fully understand its regulation.

Basolaterally applied ATP is established to inhibit Na\(^+\) and Cl\(^-\) absorption substantially (approx. 25\%) via P2X receptors (Marques et al. 2012). The current results indicate that inhibition of transport, irrespective of the mode of induction, associates with an intracellular alkalization caused by increased H\(^+\) secretion.
The main motivation for this study was to investigate the underlying signalling mechanism that explains how basolateral P2X receptor stimulation inhibits Na\(^+\) and Cl\(^-\) transport in the TAL. Currently, it was unresolved whether alterations of pH\(_i\) could matter in this signalling cascade. The current through the ROMK channel is known to be significantly reduced by intracellular acidification, which potentially could lead to transport inhibition, much similar to that observed with luminal Ba\(^{2+}\). Clearly, our results show that P2X receptor stimulation does not cause an intracellular acidification and therefore argues against this hypothesis.

Several experimental observations argue against a critical role of intracellular acidification as important modulator of Na\(^+\) and Cl\(^-\) absorption in the TAL. We show here that P2Y\(_2\) receptor and CaSR stimulation resulted in a small intracellular acidification. It is, however, unlikely that this acidification results in ROMK closure and transport inhibition, as it has been shown that acute basolateral P2Y\(_2\) receptor stimulation has no effect on Na\(^+\) and Cl\(^-\) reabsorption in the TAL (Marques et al., 2013). In accordance with this, inhibition of apical Na\(^+\)/H\(^+\) exchange with amiloride, which is known to cause marked intracellular acidification in the TAL, does not change the transepithelial voltage in perfused mouse and rat mTAL (Good 1985). These results again suggest that the pH\(_i\) sensitivity of ROMK may not play an important role in regulation of the transepithelial transport in TAL.

In summary, this study reports the novel finding that stimulation of basolateral P2X receptors causes a substantial intracellular alkalinization in the isolated perfused mouse mTAL. Together with previous studies (Good 1990, de Brujin et al., 2013), the cellular mechanism is described and highlights that the intracellular alkalinization is mediated through an increased apical NHE3 activity. This increased NHE3 activity causes H\(^+\) secretion in the mTAL and provides further support that the TAL is a site of urinary acidification.

Conflicts of interest

None.

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