Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 359 (2007) 438–444
BBRC
www.elsevier.com/locate/ybbrc

Apical P2XR contribute to $[Ca^{2+}]_i$ signaling and I_{sc} in mouse renal MCD

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Received 12 May 2007
Available online 4 June 2007

Abstract

We examined P2X receptor expression and distribution in the mouse collecting duct (CD) and their functional role in Ca^{2+} signaling. Both $P2X_1$ and $P2X_4$ were detected by RT-PCR and Western blot. Immunohistochemistry demonstrated apical $P2X_1$ and $P2X_4$ immunoreactivity in principal cells in the outer medullary CD (OMCD) and inner medullary CD (IMCD). Luminal ATP induced an increase in Ca^{2+} signaling in native medullary CD (MCD) as measured by fluorescence imaging. ATP also induced an increase in Ca^{2+} signaling in MCD cells grown in primary culture but not in the presence of P2XR antagonist PPNDS. Short circuit current (I_{sc}) measurement with mouse IMCD cells showed that P2XR agonist BzATP induced a larger I_{sc} than did P2YR agonist UTP in the apical membrane. Our data reveal for the first time that $P2X_1$ and $P2X_4$ are cell-specific with prominent immunoreactivity in the apical area of MCD cells. The finding that P2XR blockade inhibits ATP-induced Ca^{2+} signaling suggests that activation of P2XR is a key step in Ca^{2+}-dependent purinergic signaling. The result that activation of P2XR produces large I_{sc} indicates the necessity of P2XR in renal CD ion transport.
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Keywords: $P2X_1$; $P2X_4$; Calcium signaling; Short circuit current; IMCD; OMCD; Renal ion transport; Kidney; Microperfusion

There is a growing appreciation for extracellular ATP in the regulation of renal physiology including microvascular function, tubuloglomerular feed-back response, and tubular ion transport [1–4]. However, the role of purinergic signaling in the kidney, in general and in the renal CD, in particular, are largely unknown [5–7].

Two subtypes of P2 purinoceptors at the cell membrane are activated by extracellular ATP: P2Y receptors (P2YR) and P2X receptors P2XR [8]. The P2YR are coupled to G protein-linked signaling pathways. Activation of P2YR by ATP or analogs usually results in an increase of inositoltriphosphate production and causes a release of intracellular Ca^{2+}. Previous studies demonstrated that activation of P2YR in the renal CD is associated with inhibition of arginine vasopressin-stimulated water permeability in the IMCD [9,10] and inhibition of Na^+ absorption in the cortical CD (CCD) [11].

The P2XR are ligand-gated ion channels that allow cation Ca^{2+} influx [12]. Growing evidences indicate that P2XR play a key role in health and disease [13]. In particular, both $P2X_1$- and $P2X_4$-deficient mice have significantly higher blood pressure than their wild-type mouse counterparts, respectively [14,15]. Previously, we demonstrated that the mouse IMCD-3 cell line has $P2X_1$ and $P2X_4$ (in addition to P2YR) [16,17]. Intriguingly, our data also revealed that ATP failed to induce a significant increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence of P2XR antagonists, suggesting that P2XR-related signaling pathways could interact with P2YR-related signaling pathways and therefore affect P2YR-mediated cellular function [16]. However, this novel mechanism has not been tested in the native CD. Furthermore, knowledge of the distribution and localization of P2XR in the native CD is incomplete [7,18].

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2007.05.143

In this report, we investigated the expression and localization of $P2X_1$ and $P2X_4$ receptors in the mouse MCD by immunoblotting and immunohistochemistry. We examined intracellular Ca^{2+} response to luminal application of ATP and characterized the cellular response in the presence of a P2XR antagonist. Finally, we conducted a functional study of measuring I_{sc} by apical activation of P2XR or P2YR.

Materials and methods

Animals and tissue preparations. Male/female C57BL/6J and 129/SvJ mice were fed with a normal diet with free access to tap water. The animals were euthanatized, both kidneys taken and cut into 1–2 mm slices. Renal cortex (CTX), outer medulla (OM), and inner medulla (IM) were separated from the slices and kept in liquid nitrogen; then frozen at −80 °C for later protein analysis.

Reverse transcription (RT)-PCR and immunoblot analyses. Total RNA from mouse kidney was isolated for RT-PCR analysis. Specific primers (400 nM; Genomechanix, Gainesville, FL) used to amplify a fragment of mouse $P2X_1$ (NM_008771.2) were designed to anneal to base pairs (bp) 894–913 (5′-GAGAGTCGGGCCAGGACTTC-3′) on the sense strand and bp 1107–1126 (5′-GCGAATCCCAAACACCTTGA-3′) on the antisense strand (size of 233 bp). The primers for amplifying mouse $P2X_4$ (NM_011026.1) were designed to anneal to bp 382–406 (5′-AGAGA TTCCTGATAAGACCAGCATT-3′) on the sense strand and bp 1032–1057 (5′-GTCCCGGTAGTAGTATCTCTTCTTCA-3′) on the antisense strand (size of 676 bp). The specific bands of PCR products were sent for sequencing (DNA Sequencing Core Laboratory, Gainesville, FL). Mouse CTX, OM and IM proteins were isolated for standard Western blot analysis.

Tissue preparation for immunolocalization of $P2X_1$ and $P2X_4$. Adult 129/SvJ mice were anesthetized using intraperitoneal Na-pentobarbital; in later experiments, C57BL/6 mice were anesthetized with inhalant isoflurane. The kidneys were preserved by *in vivo* cardiac perfusion with PBS, pH 7.4, followed by 2% paraformaldehyde–lysine-periodate, and then immersed in fixative for 24–48 h at 4 °C. For light microscopy, transverse slices of kidney from each animal were embedded in polyester wax. Sections ~3-μm-thick were mounted on triple gelatin-coated glass slides.

The sections were dewaxed in ethanol, rehydrated, and rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in water for 30 min. The sections were rinsed with PBS, treated for 15 min with 10% normal goat serum in PBS, then incubated at 4 °C overnight with the primary antibody, diluted in PBS 1:200 for optimal $P2X_1$ localization and 1:100 for optimal $P2X_4$ localization. The sections were washed in PBS, incubated for 30 min with biotinylated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) diluted 1:250 in PBS, and again washed with PBS. The sections were treated for 30 min with the avidin-biotin complex reagent (Vector Elite, Vector Laboratories), rinsed with PBS, and then exposed to diaminobenzidine for 5 min. The sections were washed in distilled water and dehydrated with ethanol, and then xylene, mounted using Permount (Fisher Scientific), and observed by light microscopy via 100× oil-immersion objective.

Mouse MCD isolation and microperfusion. Mice were euthanatized by intraperitoneal injection of Na-pentobarbital (120 mg/kg) and cervical dislocation. Both kidneys were quickly removed, and ~1 mm coronal slices were placed in a chilled Petri dish containing a Hepes-buffered Ringer's solution (in mM: 135 NaCl, 5 KCl, 1 $CaCl_2$, 1.5 $MgCl_2$, 10 Glucose, 10 Hepes). A single MCD (or IMCD) was hand-dissected at 4 °C within 60 min and transferred to a thermostatically controlled channel style perfusion chamber (Vestavia Scientific, Vestavia Hills, AL) where both ends were aspirated into hand-fashioned holding pipettes. One end was cannulated with the perfusion pipette containing a Hepes-buffered Ringer's solution. The bath solution was continuously exchanged at a rate of 2.5 ml/min at 22 °C. The tubule was equilibrated under these conditions for dye loading and for data recording.

Fura-2/AM loading and $[Ca^{2+}]_i$ measurement. Fura-2/AM (10 μM; Molecular Probes) was loaded into MCD from the basolateral Ringer's solution for 40–60 min. Fluorescence measurements were made as described previously [16]. For experiments with primary culture cells from mouse MCD, manually dissected MCD tubules were placed on glass cover slips that were previously coated with Cell-Tak (Collaborative Biomedical Products). Cells were usually grown out from both ends of the tubules, and they were kept in culture to be used from 2 to 7 days.

Short-circuit current (I_{sc}) measurement. mIMCD-3 cells were grown on the Snapwell (growth area ~1 cm^2; Corning Costar) for 3–8 days under normal culture conditions [16]. A monolayer of cells on the Snapwell was placed into a modified vertical Ussing chamber (Harvard apparatus), and a four-electrode voltage clamp (VCC MC6, Physiological Instruments) was used to measure I_{sc}. Bath solutions were maintained at 37 °C through a warm water circulating system. Current signal was digitized by an A/D & D/A board and stored using a PC computer using Axotape 2.0 software (Axon Instruments).

Data analyses. All the fluorescence data were expressed as R/R_0, where R is the fluorescence ratio change over time and R_0 is the averaged fluorescence ratio for a baseline of 2 min; analyzed and plotted by Origin 6.0 (Microcal Software). The results are expressed as means ± SEM. Data compared from two groups were examined by paired or unpaired Student's t test. Differences were considered statistically significant at $P < 0.05$.

Results

$P2X_1$ and $P2X_4$ mRNA and protein expression in the mouse kidney

We performed RT-PCR experiments and the expected 233-bp band for $P2X_1$ and 676-bp band for $P2X_4$ were observed in the mouse kidney (Fig. 1A). The sequencing results from RT-PCR confirmed the expression of both $P2X_1$ and $P2X_4$ (data not shown). Western blot analysis showed cross-reactivity of both $P2X_1$ (50–55 kDa) and $P2X_4$ (55–60 kDa) in mouse kidney (Fig. 1B). The electrophoretic mobility of these two bands are similar to those provided by Alomone Labs and to those reported previously [16,17,19–22]. The specificity of the band was demonstrated by peptide-blocking experiments. We further investigated the distribution of $P2X_1$ and $P2X_4$ receptors in the mouse kidney by Western blotting. Immunobands of both $P2X_1$ and $P2X_4$ were present in the CTX, OM, and IM segments of the mouse kidney (Fig. 1C). The bands were also blocked by the antigen peptide.

P2XR localization by immunohistochemistry

The same antibodies that were used for protein detection in Fig. 1 were used in the immunolocalization studies. $P2X_1$ immunoreactivity was observed in a discrete band in the apical region of a subpopulation of cells in the outer stripe of the outer medullary CD ($OMCD_o$), inner stripe of the OMCD ($OMCD_i$), and inner section of the IMCD ($IMCD_i$) (Fig. 2A–C). $P2X_1$-positive cells were rare in the CCD. In these segments, the prevalence of labeled cells and their morphologic appearance suggested that they were principal cells. In the initial connecting duct (ICT), faint