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Ontogeny of Purinergic Receptor-Regulated Ca^{2+} Signaling in Mouse Cortical Collecting Duct Epithelium

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Key Words
Intracellular calcium • Purinoceptors • L-type calcium-channel • Epithelium embryology

Abstract
Changes in ATP-induced increase in $[Ca^{2+}]_i$ during collecting duct ontogeny were studied in primary monolayer cultures of mouse ureteric bud (UB) and cortical collecting duct (CCD) cells by Fura-PE3 fluorescence ratio imaging. In UB (embryonic day E14 and postnatal day P1) the ATP-stimulated increase ($EC_{50} \approx 1$ µM) in fluorescence ratio (ΔR_{ATP}) was independent of extracellular Ca^{2+} and insensitive to the P2 purinoceptor-antagonist suramin (1 mM). From day P7 onward when CCD morphogenesis had been completed ΔR_{ATP} increased and became dependent on extracellular Ca^{2+}. This ATP-stimulated Ca^{2+} entry into CCD cells was non-capacitative and suramin (1 mM)-insensitive, but sensitive to nifedipine (30 µM) and enhanced by Bay K8644 (15 µM), a blocker and an agonist of L-type Ca^{2+} channels, respectively. Quantitative RT-PCR demonstrated similar mRNA expression of L-type Ca^{2+} channel α1-subunit, $P2Y_1$, $P2Y_2$, and $P2X_{4b}$ purinoceptors in UB and CCD monolayers while the abundance of $P2X_4$ mRNA increased with CCD morphogenesis. In conclusion, both embryonic and postnatal cells express probably $P2Y_2$-stimulated Ca^{2+} release from intracellular stores. With development, the CCD epithelium acquires ATP-stimulated Ca^{2+} entry via L-type Ca^{2+} channels. This pathway might by mediated by the increasing expression of $P2X_4$-receptors resulting in an increasing ATP-dependent membrane depolarization and activation of L-type Ca^{2+} channels.

Introduction

The metanephric kidney develops from two distinct embryonic primordial precursors, the mesenchymal blastema (which generates the nephron from glomerulus to distal tubule), and the epithelial cells of the ureteric bud (UB) that give rise to the cortical collecting duct system (CCD) by branching morphogenesis [13]. Formation of the metanephric kidney in mouse is initiated at embry-

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onic (E) day E10.5 when UB invades the mesenchymal blastema. The first generation of nephrons becomes functional around birth while branching morphogenesis of the collecting duct and tubulogenesis of the last nephron generation are completed around postnatal (P) day P7. During UB-to-CCD morphogenesis principal cells acquire the vectorial Na^+ reabsorption by increasing expression of apical Na^+ and K^+ channels [16, 18].

Various subsets of P2X (ionotropic) and P2Y (metabotropic) purinoceptor subtypes are expressed along the entire nephron [3, 5]. Specificly, in collecting duct epithelium apical or basolateral expression of $P2Y_1$, $P2Y_2$, $P2X_3$, and PX_4 has been demonstrated [5, 7, 25, 30, 33]. Binding of ATP to purinoceptors leads to a rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) [5, 9], activation of protein kinase C [2, 26], or stimulation of adenylate cyclase activity via phospholipase A_2-mediated arachidonic acid release [10, 39] suggesting multiple cellular functions targeted by ATP.

In the mature distal nephron, extracellular ATP counteracts the reabsorption of water, Na^+ and Ca^{2+} [6, 26, 30, 33] suggesting a general inhibitory effect of ATP on ion and water transport [12]. While this ATP-induced transport inhibition has been shown to be independent of $[Ca^{2+}]_i$ [23, 26], other functions of renal epithelia such as cell proliferation and regulatory- and apoptotic cell volume decrease (RVD and AVD, respectively) involve $[Ca^{2+}]_I$ signaling [27-29] and are at least partially stimulated by extracellular ATP [20, 31].

To investigate this ATP-induced Ca^{2+} signaling in CCD ontogeny, primary monolayer cultures grown from UB (E14 to P1) and CCD (P7 to P20) developmental stages were studied by Fura-PE3 fluorescence imaging microscopy.

Materials and Methods

Primary monolayer cultures

Changes in the ATP-induced increase in $[Ca^{2+}]_i$ during collecting duct ontogeny were studied in primary monolayer cultures of mouse ureteric bud (UB) and cortical collecting duct (CCD) as described [18]. Cultures were grown for 2-5 days in nephron culture medium [14] supplemented with 10% fetal calf serum (FCS) and bovine pituitary extract (50 µg/ml; Sigma-Aldrich, Deisenhofen, Germany). Culture medium was exchanged daily. Within 1-3 days the cells formed confluent monolayers. Culture medium was replaced 24 h before an experiment by medium containing dexamethasone (1 µM; Sigma) instead of FCS since glucocorticoids have been demonstrated to induce branching morphogenesis *in vivo* by regulating TGF-beta 2 and TGF-beta 3 mRNA expression [21]. Furthermore, mRNA and protein expression of glucocorticoid-induced genes has been reported in embryonic mouse metanephrogenesis [17].

Quantitative RT-PCR

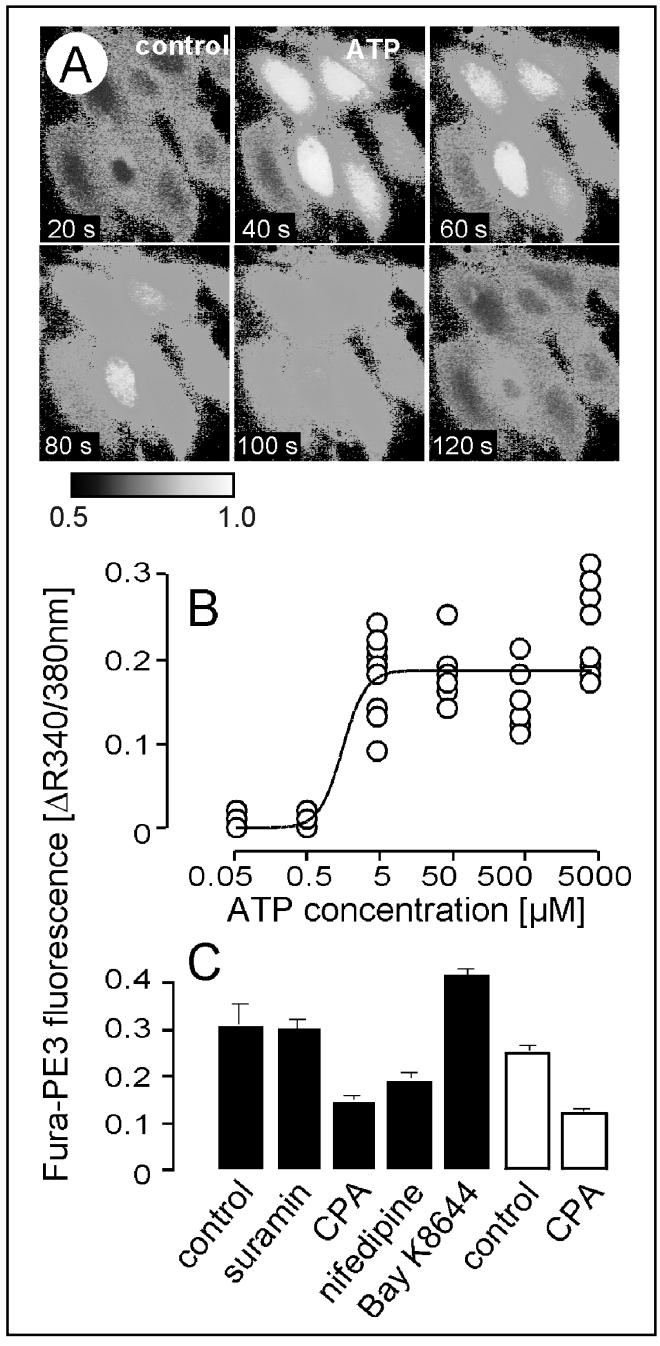
In confluent UB and CCD monolayers development-dependent mRNA expression of the purinoceptors $P2Y_1$ (GenBank accession # U22829), $P2Y_2$ (# L14751), and $P2X_4$ (# U83993), and of the L-type Ca^{2+} channel α_1-subunit L01776) was compared with the mRNA abundance of β-actin (# X00351) by quantitative RT-PCR according to [15]. Primers used were as follows: $P2Y_1$ sense: 5′-CTG GGA CTC GGA AAA ACA AA-3′ (position: 606-625), $P2Y_1$ antisense: 5′-AAG TGG CAT AAA CCC TGT CG-3′ (position: 928-947); $P2Y_2$ sense: 5′-TCT GCT TTC TGC CTT TCC AC-3′ (position:1036-1055), $P2Y_2$ antisense: 5′-TCC GTC TTG AGT CGT CAC TG-3′ (position: 1325-1344); $P2X_4$ sense: 5′- TGG CTA CAA TTT CAG GTT TGC-3′ (position 1179-1199), $P2X_4$ antisense: 5′- ACC CT G CTC GTA ATC TTC CA-3′ (position 1433-1452); L-type sense: 5′-CGA GTT TGG TTG AGC ATC AC-3′ (position: 8477-8496), L-type antisense: 5′-CTC GTG GGA CAG AAA AAT GC-3′ (position 8806-8825); β-actin sense: 5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′ (position: 384-413), β-actin antisense: 5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′ (position: 705-734). $P2X_4$-specific primers yielded a second product of 208 bp length, corresponding to alternative splicing of exon 10 ($P2X_{4b}$, GenBank accession # AF146516). The identity of all cDNA-fragments was confirmed by sequencing.

Measurement of $[Ca^{2+}]_i$

Confluent UB and CCD monolayers were washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) (pH 7.2), loaded with the Ca^{2+}-sensitive fluorescence dye Fura-PE3 AM (6 µM; Mo Bi Tec GmbH, Göttingen, Germany) and incubated for 30 min at 37°C. Fura-PE3 *AM* is an analogue of the fluorescent calcium indicator Fura-2 AM with reported identical spectral properties but resistance to both leakage and compartmentalization [37]. The used Fura-PE3 AM was dissolved in dimethylsulphoxide (1 mM) supplemented with 0.02% Pluronic F172 detergent and further diluted in physiological bath solution according to [37]. Cells were rinsed and incubated with physiological (in mM: 150 NaCl, 5 KCl, 10 N-2-hydroxyethylpiperazine- N′-2-ethanesulphonic acid (HEPES), 10 D-glucose, 1.6 $CaCl_2$, 0.8 $MgCl_2$, pH 7.2) or with Ca^{2+}-free bath solution (in mM: 150 NaCl, 5 KCl, 10 HEPES, 10 D-glucose, 0.8 MgCl2, 3 ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid/EGTA, pH 7.2).

Dishes were mounted on the stage of an inverted microscope (TV 135, Zeiss, Oberkochen, Germany) equipped with 340 nm/380 nm excitation filter wheel, 470 nm cutt-off filter, and a cooled CCD-camera (Improvision, Coventry, UK). Fura-PE3 fluorescence intensity images from 5-12 cells were recorded on-line with a frequency of 0.25 Hz at 37 °C either in standing bath solution or during constant superfusion. Since there was no difference between both recording modes the standing bath preparation was preferred in most experiments to avoid cell detachment

Fig. 1. Determination of the cytosolic free Ca^{2+} concentration in primary monolyer cultures of developing collecting duct epithelium. **A.** Pseudo-grey tone images of ureteric bud cells in monolayer culture (E14) indicating the 340/380 nm ratio (*R*) of the Fura-PE3 fluorescence intensity (the calibration of the grey scale is indicated by the bar on the lower left). Images shown were shot before (*control*) or after stimulation with ATP (5 mM; *ATP*) beginning at 20 seconds after start of measurement. Peak values which occured between 15 to 30s after addition of ATP were used for data analysis throughout all experiments. **B.** Dose-dependence of the ATP-induced change of *R* (ΔR_{ATP}). For each ATP concentration ΔR_{ATP} values were recorded in unpaired experiments. Individual data of n = 8-12 ureteric bud cells (P1) are shown. **C.** Characterization of ΔR_{ATP}. Changes of *R* were elicited by stimulation with ATP (5 mM) in physiological (*closed bars*) and Ca^{2+}-free bath solution (*open bars*). ΔR_{ATP} was determined under control conditions (*control*), after pre- and coincubation with cyclopiazonic acid (0.5 mM; *CPA*), nifedipine (30 µM, *nifedipine*) and suramin (1 mM; *suramin*), respectively, or during co-administration of Bay K8644 (15 µM; *Bay K8644*). Data are from CCD monolayers cultured at developmental stage P7 (means ± SE; n = 20-35 cells).



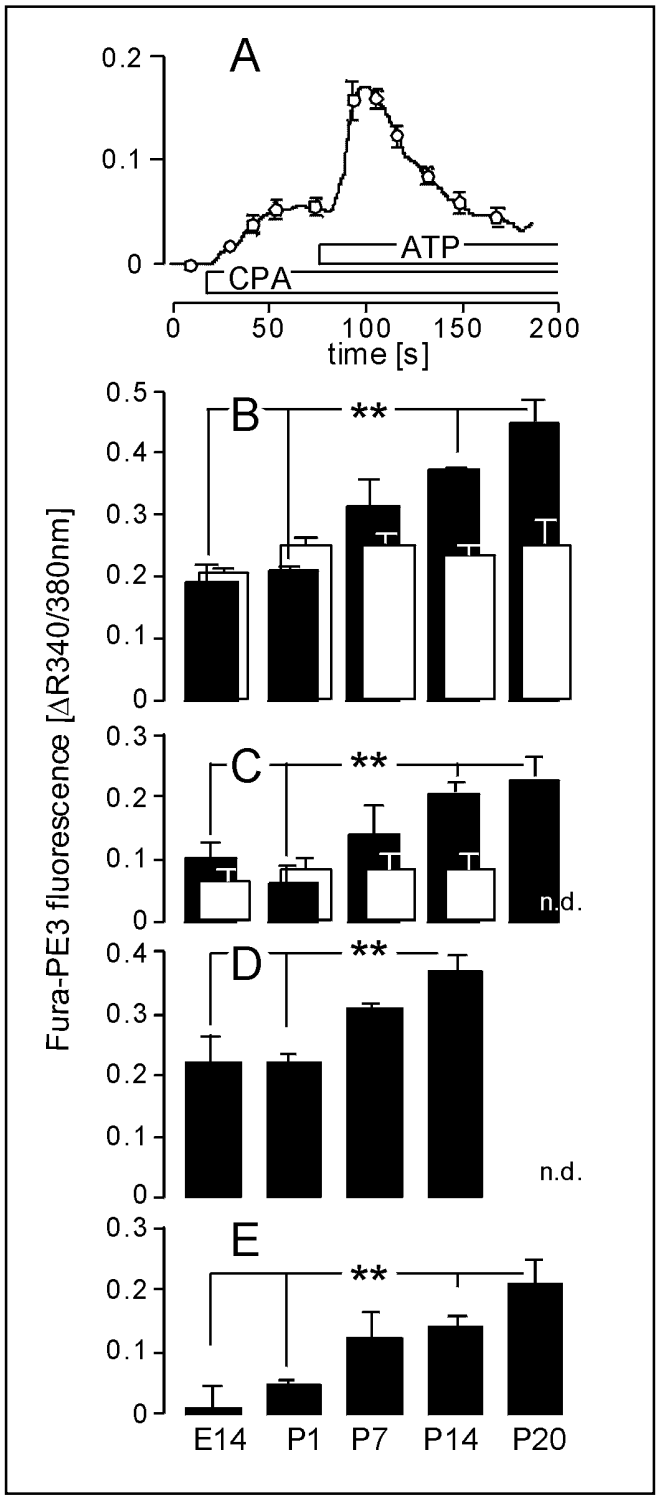
which often occured when day E14 cultures were superfused. Cells were recorded in physiological or Ca^{2+}-free bath solution before and during stimulation with ATP (Sigma). Since millimolar saturating concentrations of ATP have been reported for P2 receptor subtypes [1], a supramaximal (5 mM) concentration of ATP was used in most experiments in order to guarantee saturation of all possible subtypes of P2 receptors which might be differentially expressed in UB and CCD cells. Between 60 – 90% of cells responded to stimulation by ATP while superfusion with (or addition of) buffer solution alone did not evoke any time-dependent change in Fura-P3 fluorescence ratio (n=15). In some experiments nifedipine, cyclopiazonic acid, or suramin (all Sigma) were pre- and coincubated with ATP. In other experiments the L-type Ca^{2+} channel agonist Bay K8644 (Sigma) was coincubated with ATP.

As a measure of $[Ca^{2+}]_i$, the The Fura-PE3 fluorescence emission ratio at 340 nm/380 nm excitation was calculated with IonVision III (ImproVision, Coventry, UK) software. $[Ca^{2+}]_i$ was calculated using the following equation: $Ca^{2+} = K_D[(R - R_{min})/(R_{max} - R)]\beta$ [11], where K_D is the Fura PE3-dissociation constant for Ca^{2+} (290 nM; [37]) and β is the ratio of the emission intensity between Ca^{2+}-free and Ca^{2+}-saturated solution (3 mM Ca^{2+}), both measured at 380 nm of excitation. Calibrations were performed by cell permeabilization with ionophore A23187 (10 µM; Sigma). Auto-fluorescence was negligible, as estimated in non-dye-loaded cell monolayer.

Results

ATP-stimulated changes of $[Ca^{2+}]_i$ were studied by imaging of the Fura PE3 fluorescence in primary monolayer cultures of UB and CCD cells (Fig. 1A). Non-stimulated UB and CCD monolayers have $[Ca^{2+}]_i$ values of 85-105 nM, thus suggesting similar resting $[Ca^{2+}]_i$ during all developmental stages (not shown). Stimulation

Fig. 2. Ontogeny of ATP-stimulated $[Ca^{2+}]_i$ signaling in collecting duct epithelium. **A.** CPA (500 µM)- and ATP (5 mM)-induced changes of *R* as recorded in physiological bath solution (means ± SE of n = 8 CCD (P7) cells). **B-E.** Ontogeny of ΔR_{ATP}. ATP (5 mM)-induced increase in *R* was recorded in UB and CCD monolayers from different developmental stages (E14 to P1 and P7 to P20, respectively) . Cells were incubated in physiological (*closed bars*) and Ca^{2+}-free bath solution (*open bars*) in the absence (**B**) and presence of (**C**) CPA (0.5 mM) and (**D**) suramin (1mM), respectively. (**E**) Nifedipine-sensitive ΔR_{ATP} fraction: ATP (5 mM)-induced increase in *R* was measured in physiological bath solution in the presence of nifedipine (30 µM), subtracted from the ΔR_{ATP} control values (unpaired experiments shown in Fig. 2B, closed bars), and the nifedipine-sensitive fraction of ΔR_{ATP} was plotted against the developmental stage (means ± SE; n = 10-35 cells; **: $P \leq 0.01$, two-tailed t-test; n.d.: not determined).



by ATP induced an increase in 340/380nm fluorescence ratio (ΔR_{ATP}) with an EC_{50} in the range of 1 µM (Fig. 1B) demonstrating functional expression of purinoceptors in early collecting duct morphogenesis**.**

In order to characterize ΔR_{ATP}, the following experimental protocols were applied to both UB and CCD monolayer cultures (Fig. 1C): To differentiate between intracellular Ca^{2+}-store release and extracellular Ca^{2+}-entry, ΔR_{ATP} values were obtained in both physiological and Ca^{2+}-free bath solution, and compared. To identify the possible involvement of capacitative Ca^{2+} entry, ΔR_{ATP} was recorded after pre- / co-incubation with cyclopiazonic acid (CPA), a blocker of the Ca^{2+}-ATPase of the endoplasmic reticulum. Moreover, ΔR_{ATP} was determined in the presence of Bay K8644 or nifedipine (an agonist and an inhibitor of voltage-gated Ca^{2+} channels, respectively) or in the presence of suramin (an antagonist of the majority of cloned P2 purinoceptor subtypes such as $P2X_{1-3}$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_3$, $P2Y_6$, and subpopulations of $P2Y_2$ [32]).

When cells were bathed in Ca^{2+}-free solution, ATP (5 mM) induced similar increases in $[Ca^{2+}]_i$ in both UB (E14-P1) and CCD (P7-20) stages (Fig. 2B, open bars): $[Ca^{2+}]_i$ increased by 161 ± 4 nM (n=28 cells) and 216 ± 20 nM (n=19) in UB (E14) and CCD cells (P20), respectively. Pre-emptying the intracellular stores by pre-incubation with CPA induced a small but sustained increase of $[Ca^{2+}]_i$ (Abb. 2A). ΔR_{CPA} did not differ between the developmental stages but was slightly larger in physiological compared to Ca^{2+}-free bath solution suggestive of a capacitative Ca^{2+} entry stimulated by CPA (not shown). Pre- and co-incubation of CPA decreased ΔR_{ATP} in Ca^{2+}-free solution in all stages to about 35% (compare Figs. 2B and C, open bars) indicating that CPA and ATP induced Ca^{2+} release from the same Ca^{2+} stores.

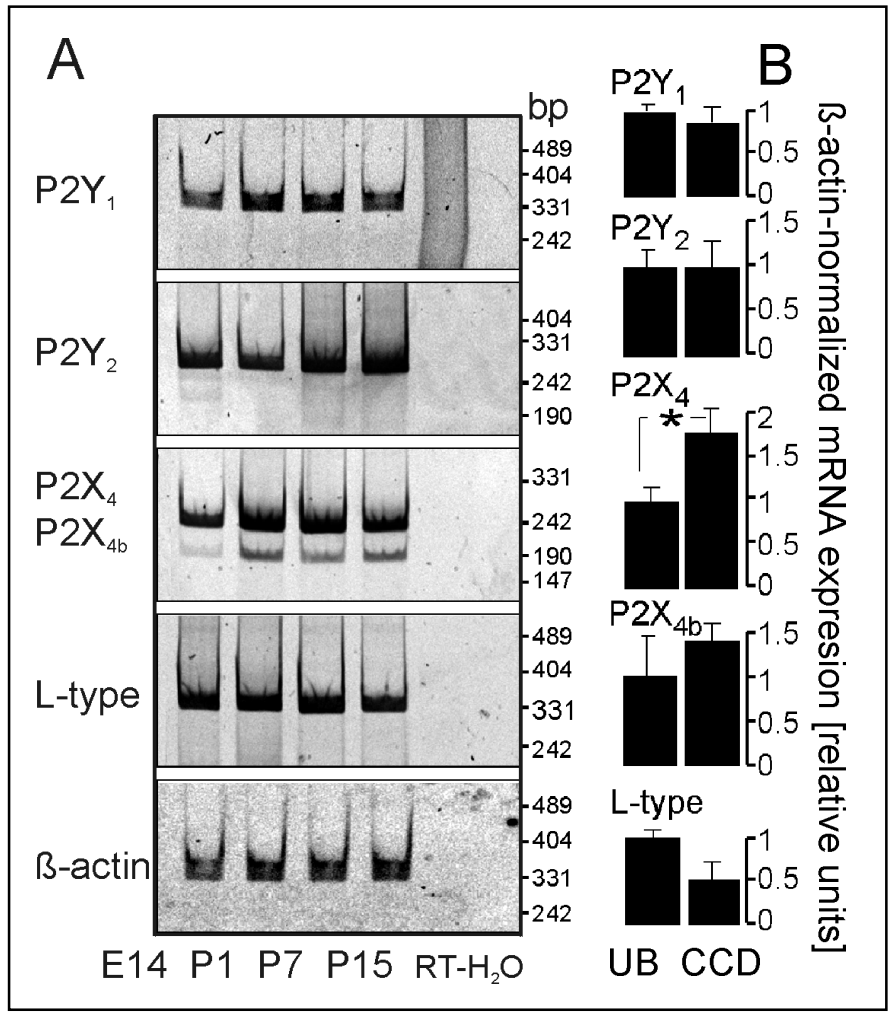


Fig. 3. mRNA expression of $P2Y_1$, $P2Y_2$, $P2X_4$, $P2X_{4-b}$ and L-type Ca^{2+} channel α_1-subunit in collecting duct development as determined by quantitative RT-PCR. **A.** Gels showing RT-PCR products specific for cDNA fragments of $P2Y_1$, $P2Y_2$, $P2X_4$, $P2X_{4b}$, L-type calcium channel α_1-subunit) (upper gels) and β-actin (lower gel), amplified from UB and CCD monolayers grown from various embryonic and postnatal developmental stages (E14-P15). In addition, reverse transcriptase-negative (*RT*–) and PCR water controls (H_20) are shown (VistaGreen stained polyacrylamide gels, visualized by a Fluorophospho-Imager). **B.** Quantified and β-actin-normalized PCR product amounts as a measure of relative mRNA expression in UB (E14-P1) and CCD (P7-P15) monolayers (means ± SE; n = 3-8; *: $P \leq 0.05$, one-tailed t-test).

ATP (5 mM) stimulation in physiological bath solution, in sharp contrast, induced $[Ca^{2+}]_i$ rises that increased with development (Fig. 2B, closed bars): $[Ca^{2+}]_i$ increased by 157 ± 8 nM (n=11) and 459 ± 35 nM (n=10) in UB (E14) and CCD cells (P20), respectively. Comparing ΔR_{ATP} in Ca^{2+}-free- with that in physiological solution indicates developmental acquisition of a ΔR_{ATP} fraction which was dependent on external Ca^{2+} starting with the first CCD stage at day P7. CPA pre- and coincubation attenuated ΔR_{ATP} in physiological bath solution (compare Figs. 2B and C, closed bars). This CPA-sensitive fraction did not differ among the developmental stages similar to the CPA-sensitive fraction of ΔR_{ATP} in Ca^{2+}-free solution.

In contrast, the CPA-insensitive fraction of ΔR_{ATP} that was dependent on extracellular Ca^{2+} (Fig. 2C, difference between open and closed bars) increased with CCD development in a similar fashion as the extracellular Ca^{2+}-dependent ΔR_{ATP} fraction in the absence of CPA. This indicated that this fraction was independent of intracellular Ca^{2+} stores and, therefore, not due to capacitative Ca^{2+} entry.

Pre- and coincubation with suramin did not alter ΔR_{ATP} in physiological bath solution in neither developmental stage (Fig. 2D) indicating that ATP-induced Ca^{2+} release from intracellular stores as well as developing ATP-induced Ca^{2+} entry was triggered by purinoceptors which were insensitive to suramin.

Bay K8644, when coincubated with ATP did not enhance ΔR_{ATP} in E14 and P1 UB monolayers, i.e., in those stages where ATP did not induce Ca^{2+} entry (not shown). Starting with the early CCD stage (P7), Bay

K8644 induced an enhancement of ΔR_{ATP} in physiological bath solution (Fig. 1C) suggesting both, functional expression of voltage-gated Ca^{2+} channels in CCD cells and involvement of these channels in ΔR_{ATP}. Accordingly, nifedipine inhibited in physiological bath solution a fraction of ΔR_{ATP} (Fig. 2E) which did not differ from the fraction dependent on extracellular Ca^{2+} (Fig. 2B) strongly suggesting that almost all of the developing ATP-induced Ca^{2+} entry occurred via voltage-gated Ca^{2+} channels.

mRNA for the L-type Ca^{2+}-channel (α_1-subunit) was detected in UB and CCD monolayers using non-saturating RT-PCR (Fig. 3A), further confirming the expression of these channels during branching morphogenesis. In addition, RT-PCRs for $P2Y_1$, $P2Y_2$ and $P2X_4$ purinoceptor were performed since these receptors are the most common purinoceptors in epithelial cells [28]. Quantification and β-actin-normalization of the PCR-products disclosed constant and slightly decreasing mRNA expression of $P2Y_1$, $P2Y_2$, and L-type Ca^{2+} channel α_1-subunit during UB-to-CCD transition, respectively, while P2X4 mRNA was significantly upregulated (Fig. 3B). $P2X_4$-specific primers also detected a novel splice variant of $P2X_4$ lacking exon 10 ($P2X_{4b,}$ GenBank accession # AF146516). The deletion of exon 10 (22 AA) in this splice variant does not result in a frame-shift, suggesting that $P2X_{4b}$ may be a functional purinoceptor.

Discussion

This work has examined the ATP-induced Ca^{2+} response of the developing mouse collecting duct epithelium in primary culture, using the Fura-PE3 fluorescence method. The principal findings are: i) in all developmental stages (E14 - P20), extracellular ATP induced a suramin-insensitive release of Ca^{2+} from intracellular stores strongly suggesting development-independent expression of metabotropic purinoceptors (probably $P2Y_2$) and their downstream signaling cascades. ii) With CCD development (stage P7 to P14) an ATP-induced suramin-insensitive but nifedipine- and BayK 8644-sensitive Ca^{2+} entry was up-regulated indicating developmental acquisition of ATP-induced activity of L-type Ca^{2+} channels. The mRNA expression of the L-type Ca^{2+} channel α_1-subunit was demonstrated in all stages by RT-PCR. iii) With UB-to CCD transition mRNA abundance of the ionotropic $P2X_4$ receptor increased.

P2X receptors mediate the rapid nonselective passage of Na^+, K^+, and Ca^{2+} and, therefore, contribute themselves to Ca^{2+} entry into the cell. However, P2X receptor-mediated depolarization of the membrane potential leads to secondary activation of voltage-dependent Ca^{2+} channels which probably represents the primary source of Ca^{2+} influx [32]. Thus, $P2X_4$ receptors of CCD might account for the activation of L-type Ca^{2+} channels observed in the present study. In addition to the present data, the expression of L-type Ca^{2+} channels has been reported in renal epithelia [38, 40] and a nifedipine- and verapamil-sensitive mechanism of Ca^{2+}-entry has been demonstrated to be involved in Ca^{2+} signaling of collecting duct cells during RVD [36].

Secretion of ATP and its autocrine action on Ca^{2+} signaling is involved in RVD and AVD [31]. The *cystic fibrosis transmembrane conductance regulator* (CFTR) modulates the ATP release to the extracellular membrane face [8, 22] and facilitates RVD via purinoceptors [4]. Conversely, ATP has been shown to stimulate CFTR in CFTR-transfected cells [35]. With development, the collecting duct epithelium acquires functional cell volume-regulatory anion channels [19] which, in concert with K^+ channels, generate RVD upon cell swelling [27]. In addition, CFTR mRNA is increasingly expressed during postnatal UB and CCD development [15]. Taken together, this might suggest that the developmental acquisition of ATP-induced Ca^{2+} entry via L-type Ca^{2+} channels reflects the increasing expression of the machinery that generates RVD.

In renal epithelia the ATP-induced inhibition of Na^+, Ca^{2+}, and H_2O reabsorption is probably mediated by $P2Y_2$ receptors [6, 24, 26, 33]. In M1 collecting duct cells ATP induces, in addition to inhibition of Na^+ reabsorption, the stimulation of Cl^- secretion. The present study shows that embryonic UB cells already express a mechanism of ATP-induced P2Y-mediated Ca^{2+} release. However these cells are yet unable to accomplish vectorial transport because of their immature epithelial polarity [18].

UB and CCD monolayers expressed $P2Y_2$ mRNA suggesting that the ATP-induced Ca^{2+} release was triggered by $P2Y_2$ purinoceptors. Further involvement of other suramin-insensitive P2Y purinoceptors such as $P2Y_4$ [34] cannot be deduced from the present study.

In summary, P2Y-induced Ca^{2+} release was early expressed in CCD ontogeny while (possibly) P2X-induced Ca^{2+} entry via L-type Ca^{2+} channels was increas-

ingly acquired. Purinoceptor-induced Ca^{2+}-release and Ca^{2+}-entry might regulate vectorial transport and cell volume, respectively. Ca^{2+}-entry was upregulated after the completion of CCD morphogenesis, prior to the onset of Na^{+} reabsorption.

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