

AVP-stimulated nucleotide secretion in perfused mouse medullary thick ascending limb and cortical collecting duct

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Odgaard E, Praetorius HA, Leipziger J. AVP-stimulated nucleotide secretion in perfused mouse medullary thick ascending limb and cortical collecting duct. *Am J Physiol Renal Physiol* 297: F341–F349, 2009. First published June 10, 2009; doi:10.1152/ajprenal.00190.2009.—Extracellular nucleotides are local, short-lived signaling molecules that inhibit renal tubular transport via both luminal and basolateral P2 receptors. Apparently, the renal epithelium itself is able to release nucleotides. The mechanism and circumstances under which nucleotide release is stimulated remain elusive. Here, we investigate the phenomenon of nucleotide secretion in intact, perfused mouse medullary thick ascending limb (mTAL) and cortical collecting duct (CCD). The nucleotide secretion was monitored by a biosensor adapted to register nucleotides in the tubular outflow. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured simultaneously in the biosensor cells and the renal tubule with fluo 4. We were able to identify spontaneous tubular nucleotide secretion in resting perfused mTAL. In this preparation, 10 nM AVP and 1-desamino-8-D-arginine vasopressin (dDAVP) induced robust $[\text{Ca}^{2+}]_i$ oscillations, whereas AVP in the CCD induced large, slow, and transient $[\text{Ca}^{2+}]_i$ elevations. Importantly, we identify that AVP/dDAVP triggers tubular secretion of nucleotides in the mTAL. After addition of AVP/dDAVP, the biosensor registered bursts of nucleotides in the tubular perfusate, corresponding to a tubular nucleotide concentration of $\sim 0.2\text{--}0.3\ \mu\text{M}$. A very similar response was observed after AVP stimulation of CCDs. Thus AVP stimulated tubular secretion of nucleotides in a burst-like pattern with peak tubular nucleotide concentrations in the low-micromolar range. We speculate that local nucleotide signaling is an intrinsic feedback element of hormonal control of renal tubular transport.

ATP release; renal tubule; vasopressin; purinergic

THE RENAL TUBULAR EPITHELIUM displays widespread expression of luminal and basolateral purinergic (P2) receptors (1, 17, 34). P2 receptor stimulation most commonly inhibits ion transport along the entire renal tubule, a phenomenon best documented for aquaporin-2 (AQP2)- or epithelial Na^+ channel (ENaC)-mediated water or Na^+ transport in the distal tubule (13, 16, 27). Global knockout of one of the prominent renal epithelial P2 receptors (P2Y₂) is associated with increased urinary concentration and elevated blood pressure (30). This supports the notion that renal transport could be under tonic purinergic inhibition and that removal of one of the system's essential components causes increased absorption of water and Na^+ , for example. A substantial amount of data supports that the epithelial cells are able to release nucleotide in sufficient quantities to induce local nucleotide signaling (15, 29, 38). Several studies indicate that renal epithelia constitutively secrete ATP, which is consistent with a tonic purinergic inhibition of renal epithelial transport (6, 25, 38). The mechanism and regulation

of this ATP secretion from epithelia are still incompletely understood, and we recently reviewed this phenomenon in more detail (29). In search of the elements involved in renal epithelial ATP release, we recently discovered that an increase in tubular flow apparently triggers the release of nucleotides and auto- and paracrine activation of tubular P2 receptors (10). Subsequent studies support these observations in cultured renal and also bile duct epithelia (40, 42). These results could signify that tubular flow changes per se modulate transport (28).

In this study, we travel a novel path to understand the role of intrarenal purinergic signaling. Several studies have indicated that G protein-coupled receptor (GPCR) stimulation is able to trigger a substantial nucleotide release from very different cell preparations. To mention a few, nucleotide release is induced by thrombin from platelets (20), by muscarinic agonists from astrocytes (11), by lysophosphatidic acid from endothelial cells (8), or by P2 receptor stimulation from Swann cells (19). Numerous observations support that an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is required for this response (29). Recently, we reported that cultured and freshly isolated renal epithelia display significant spontaneous $[\text{Ca}^{2+}]_i$ oscillations that reflect release of nucleotides (6). In this context, it is noteworthy that low concentrations of AVP are able to induce $[\text{Ca}^{2+}]_i$ oscillations in the isolated, perfused inner medullary collecting duct (43). Based on this, we hypothesized that “classic” hormones that regulate renal transport and increase renal epithelial $[\text{Ca}^{2+}]_i$ may trigger nucleotide release from renal tubules. In this study, we chose AVP, the indispensable hormone for the activation of tubular water, urea, and to a lesser degree also Na^+ transport. AVP increases intracellular cAMP via V2 receptor activation, which then induces vesicular trafficking to and fusion with the luminal membrane (14). Furthermore, AVP also provokes $[\text{Ca}^{2+}]_i$ increases in the thick ascending limb and along the collecting duct system, either as $[\text{Ca}^{2+}]_i$ transients (4, 5, 12) or as $[\text{Ca}^{2+}]_i$ oscillations (43). In this study, we focus on the question of whether AVP stimulation is able to induce tubular nucleotide secretion, i.e., the stimulated appearance of nucleotides in the luminal fluid compartment. For this purpose, we used hP2Y₂ receptor cells expressing 132-1N1 cells as biosensors positioned directly in the outflow of isolated, perfused mouse medullary thick ascending limbs (mTAL).

Here, we describe spontaneous tubular nucleotide secretion in the mTAL and AVP-stimulated nucleotide secretion in the mTAL and cortical collecting duct (CCD). The findings provide a new fundamental perspective of the regulation of renal transport and indicate a likely relationship of transport-activating systemic hormones like AVP and a local transport-opposing purinergic feedback system.

MATERIALS AND METHODS

Animals. Experiments were performed using 4- to 7-wk-old mice of either sex. Animals had free access to standard rodent diet and tap

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water. Mouse handling complied with Danish animal welfare regulations. In this study, mice on a mixed genetic background (B6D/2SV129) were used.

Tubule perfusion. Mice were killed by decapitation. Both kidneys were removed and placed in ice-cold dissection solution (see below) before being sliced, as previously described (41). Slices were transferred to a dissection chamber cooled to 4°C and gassed with 5% CO₂-95% O₂. mTAL were isolated in dissection solution (see *Solutions and chemicals*) from the inner stripe of the outer medulla using fine forceps. Kidney tubules were transferred to a perfusion chamber on an inverted microscope. Isolated tubules were perfused by a system of concentric glass pipettes as described previously (7). Utmost care was taken to select only viable tubules. The tubule was placed directly on the glass bottom of the experimental chamber and perfused from one side, leaving the other end open for free outflow of the tubular perfusate. An additional holding pipette was often used to stabilize the preparation.

Video imaging of perfused renal tubules and biosensor cells. The set-up comprised an inverted microscope (Axiovert 100TV, Zeiss, Jena, Germany) with the objective LD LCI Plan-Apochromat (Oil, Glyc, water), 25 × 0.8 (Zeiss), a monochromator (Polychrome IV, Till Photonics, Planegg, Germany), and a CCD camera (MicroMax, 5 MHz, Princeton Instruments, Trenton, NJ). Image acquisition and data analysis were performed with Metamorph/Metafluor (Universal Imaging, West Chester, PA). Measurement of [Ca²⁺]_i was performed with fluo 4. Tubules were incubated in 20 μM basolateral fluo 4-AM for 20 min at room temperature in control solution. The fluo 4-measured [Ca²⁺]_i changes (excitation: 488 nm, emission: >510 nm) were expressed relative to stable baseline values (acquisition frequency: 0.5 Hz). To reduce potential photo damage (39), the excitation light was controlled by neutral-density filters in the excitation light path and a 8 × 8 binning to reduce the exposure time. Fluorescence was recorded from the entire tubule, and the biosensor cells were positioned in the same optical plane. During dye loading, the tubule was continuously perfused. The experiment was started 5–10 min after washout of fluo 4 dye. For each experiment, it was important to know that the tubule was well perfused and, more importantly, that the fluid leaving the tubule actually engulfed the biosensor cells. This was achieved by adding 5 μM fura 2 potassium salt into the perfusion solution and imaging this fluorescence flow signal at the beginning and the end of the experiment. With appropriate excitation, the fura 2 fluorescence exhaust from the tubule is easily visible and the position of the biosensor cell holding pipette can be adjusted appropriately. The inclusion of luminal fura 2 potassium salt did not interfere with the cellular fluo 4 fluorescence signal. During the experiments, the entire preparation was superfused at ~3–5 ml/min, i.e., ~10 bath exchanges/min.

Cell culture. 132-1N1 cells with or without stably transfected hP2Y₂ receptors were grown in DMEM with 10% FBS (GIBCO, Grand Island, NY) and 2 mM glutamine but without riboflavin and with antibiotics (1 U/ml penicillin and 100 μg/ml streptomycin). The human P2Y₂ receptor-expressing 132-1N1 cells were kindly provided by Robert Nicholas (Chapel Hill, NC).

Solutions and chemicals. Experiments were performed at 37°C with the following control solution: (in mM) 145 NaCl, 1 MgCl₂, 1.3 Ca-gluconate, 5 D-glucose, 0.4 KH₂PO₄, 1.6 K₂HPO₄, and 5 HEPES. The dissection solution contained (in mM) 118 NaCl, 24 NaHCO₃, 1 MgCl₂, 1.3 Ca-gluconate, 5 D-glucose, 0.4 KH₂PO₄, and 1.6 K₂HPO₄. Solutions were titrated with NaOH to pH 7.4 (37°C).

Fluo 4-AM was obtained from Invitrogen (Taastrup, Denmark). All other chemicals were obtained from Sigma-Aldrich Denmark (Valensbaek, Denmark) and Merck (Darmstadt, Germany). Agonist solutions were prepared directly before the experiment.

Statistics. Data are shown as means ± SE. For the experimental series, *n* reflects the number of tubules used. On average, two tubules were used from each mouse. Paired and unpaired *t*-tests were used to

compare mean values within one experimental series. A *P* value of <0.05 was accepted to indicate statistical significance.

RESULTS

AVP- and 1-desamino-8-D-arginine vasopressin-induced [Ca²⁺]_i oscillations in perfused mTAL. The primary aim of this project was to investigate whether AVP stimulation of the mTAL and CCD activates tubular nucleotide secretion. First, we investigated the effect of vasopressin (AVP) and 1-desamino-8-D-arginine vasopressin (dDAVP) as [Ca²⁺]_i agonists in mouse mTAL. Figure 1 shows that AVP and dDAVP (10 nM) consistently induces [Ca²⁺]_i increases in mTAL. Importantly, these hormone-induced [Ca²⁺]_i increases were composed of many local, asynchronous [Ca²⁺]_i elevations, which appeared to originate from single tubular cells. The original [Ca²⁺]_i traces in Fig. 1, *A* and *D*, were derived from the matching regions of interest depicted in the figure inset and comprise ~5–10 cells. As previously published, nonstimulated, isolated perfused mTAL segments display spontaneous [Ca²⁺]_i oscillations. This is visible in the unsteady baseline in Fig. 3*A* before the addition of AVP. After AVP/dDAVP, these [Ca²⁺]_i oscillations were greatly increased, in both amplitude (Fig. 1, *A* and *D*) and frequency (Fig. 1, *C* and *F*). Importantly, the maximal [Ca²⁺]_i increase in response to AVP/dDAVP showed a delay of 20–40 s (Fig. 1, *B* and *E*). In summary, these data provide clear evidence for AVP/dDAVP-induced [Ca²⁺]_i elevations in mouse mTAL, which are composed of multiple regional [Ca²⁺]_i oscillations. No apparent differences were observed between the effects induced by AVP and dDAVP.

132-1N1 cell as ATP biosensor cells for nucleotide secretion from renal tubules. 132-1N1 cells are known to not express P2 receptors (26). These cells become strongly responsive to extracellular nucleotides when they are transfected with different P2 receptors (26). In this study, we used 132-1N1 cells stably transfected with hP2Y₂ as ATP/UTP biosensors. P2Y₂ receptor-expressing 132-1N1 cells were used 1–2 days after trypsinization. Non-P2 receptor-expressing 132-1N1 cells served as controls. Cells that had not fully settled to the bottom of the culture dish after splitting were transferred to the experimental bath chamber. hP2Y₂-132-1N1 cell clusters of two to four cells were picked up by subtle suction via a fire-polished holding pipette. Excess cells were washed away. In Fig. 3*A*, two biosensor cells can be seen attached to a holding pipette. The hP2Y₂-132-1N1 cells were tested for their responsiveness to extracellular ATP and UTP. The results are shown in Fig. 2, *A* and *B*, as an original trace of UTP responses (from 0.01 to 100 μM, *left*) and the concentration-response relationship of the various series. Note that hP2Y₂-132-1N1 cells respond to concentrations <0.2 μM UTP/ATP with significant elevations of [Ca²⁺]_i (EC₅₀ UTP: 0.26 μM, EC₅₀ ATP: 0.4 μM). The nontransfected 132-1N1 cells did not show any nucleotide-stimulated elevations in [Ca²⁺]_i. In Fig. 2*A*, extracellular ATP/UTP was applied for longer periods (60–90 s), and it may be questioned whether the hP2Y₂-132-1N1 biosensor cells are able to detect short applications of ATP/UTP. This was investigated with a custom-built, very fast fluid application system that consisted of a double-barreled glass pipette fabricated on a glass microforge (tip diameter ~50 μm). Each barrel was equipped with a fine polyethylene plastic catheter to administer either control or nucleotide-containing solution (1 μM). The

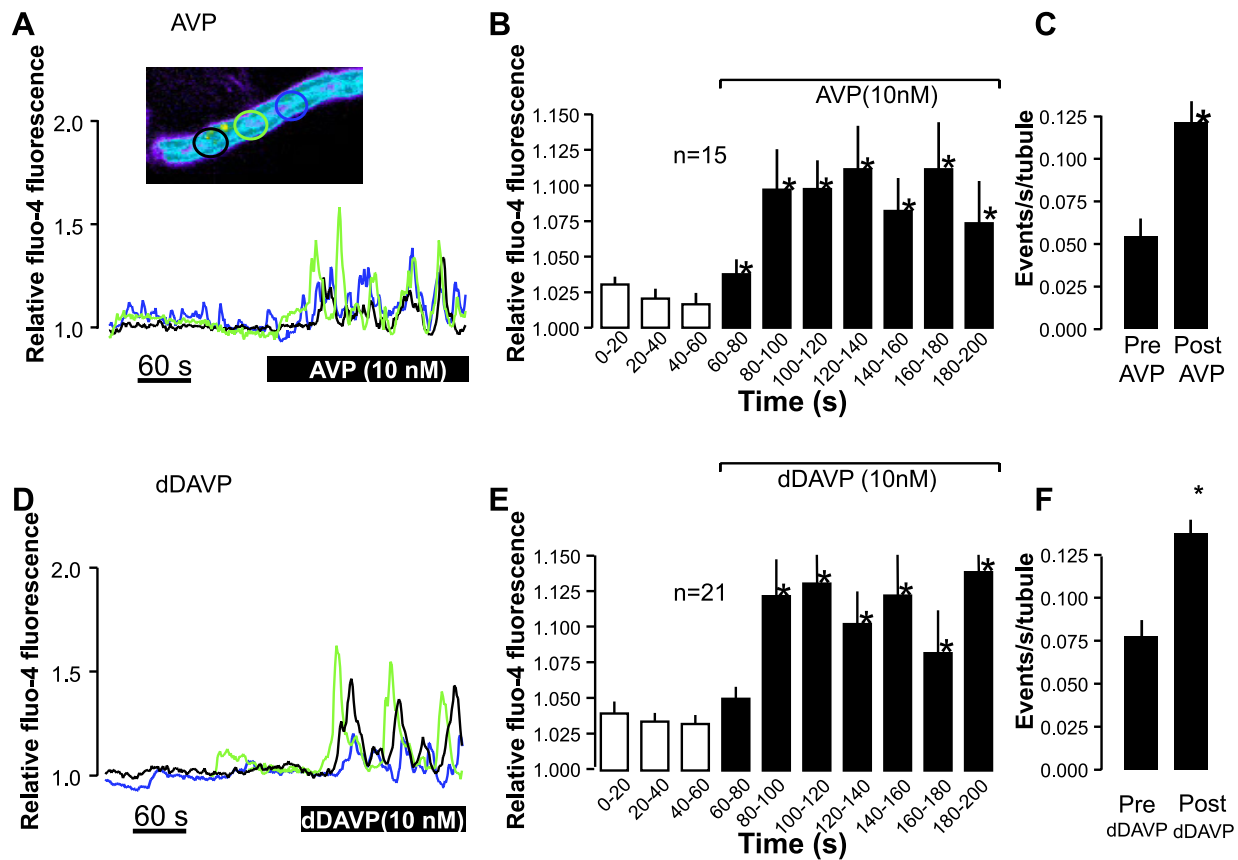


Fig. 1. AVP- and 1-desamino-8-D-arginine vasopressin (dDAVP)-induced intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) oscillations in isolated, perfused mouse medullary thick ascending limb (mTAL). Original fluo 4 recordings are shown of 3 regions of interest (left; see fluorescence inset) placed over ~5–10 cells (A and D). Commonly, tubular $[\text{Ca}^{2+}]_i$ signals showed some smaller oscillations before addition of AVP/dDAVP (see blue trace in A). After the addition of AVP/dDAVP, very robust oscillatory $[\text{Ca}^{2+}]_i$ increases were measured (A–F). No apparent differences were observed between the effect of AVP and dDAVP. Note the marked delay of some 20–40 s before AVP/dDAVP induced a significant $[\text{Ca}^{2+}]_i$ increase (A and D).

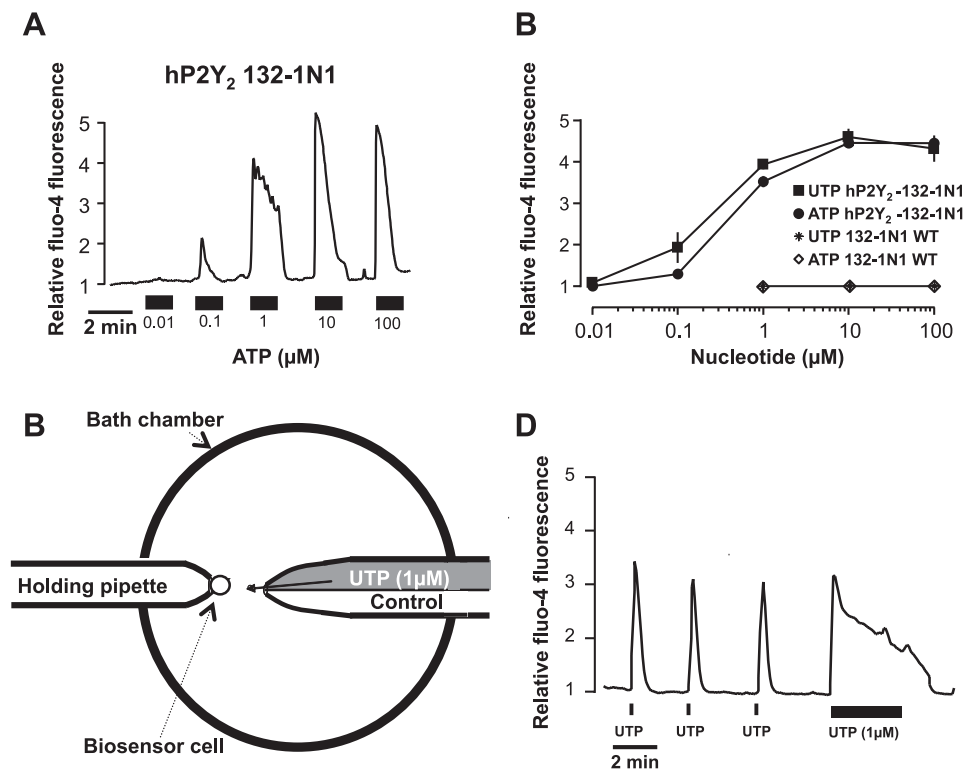
fast-application pipette was positioned close to the biosensor cells (~50–100 μm). The biosensor cells were held in the stream of control solution for several minutes. Turning the flow of control solution on and off never induced an increase in $[\text{Ca}^{2+}]_i$ in the biosensor cells. This indicates that flow per se is not a $[\text{Ca}^{2+}]_i$ -increasing stimulus in hP2Y₂-132-1N1 biosensor cells. By switching to the nucleotide-containing barrel, a very fast agonist application (on and off) could be performed. In this way, nucleotides could be applied in short pulses that lasted <1 s. As shown in Fig. 2D, right, three 1-s-long repetitive pulses of 1 μM UTP led to similar and large $[\text{Ca}^{2+}]_i$ increases in the biosensor cells. The fourth stimulus was given for ~60 s and showed a typical biphasic $[\text{Ca}^{2+}]_i$ responses known for many GPCR/G α q-mediated effects. These experiments establish that very short nucleotide stimuli trigger significant and reproducible $[\text{Ca}^{2+}]_i$ elevations in these biosensor cells. Importantly, neither hP2Y₂-132-1N1 nor the control 132-1N1 cells responded to AVP (10 nM; $n = 3$ each; data not shown).

Spontaneous tubular nucleotide secretion from perfused mTAL. Recently, we described that cultured renal epithelial cells and isolated, perfused mTAL segments display slow spontaneous $[\text{Ca}^{2+}]_i$ oscillations that reflect spontaneous nucleotide release (6). Here, we confirm these findings with the above-described technique. Specifically, we addressed whether nucleotides are spontaneously released into the lumen of the

intact, perfused tubule. To this end, the biosensor cells were used to record nucleotide outflow from a freshly isolated mTAL (Fig. 3). Initially, the biosensor cells were placed away from the tubule's outflow. The left inset below Fig. 3B depicts the positioning of a biosensor cell in relation to a perfused mTAL segment. During this initial period, the biosensor cells showed a stable baseline $[\text{Ca}^{2+}]_i$ recording. Immediately after the biosensor cells were placed in the tubular outflow, their $[\text{Ca}^{2+}]_i$ began to increase randomly well above baseline value and this behavior stopped instantaneously after the biosensors were moved away from the tubule outflow (see Supplementary video 1; all supplementary material for this article is accessible on the journal web site). This was a reproducible observation seen in eight of eight experiments. Figure 3B summarizes the mean relative fluo 4 fluorescence increase during the period the biosensor was placed in front of the tubule. Flow alone from the perfusion pipette, i.e., without the mounted renal tubule, never altered the resting $[\text{Ca}^{2+}]_i$ of the biosensor cells. These experiments indicate that mTAL segments spontaneously secrete nucleotides into their lumen.

AVP and dDAVP trigger nucleotide secretion from perfused mTAL. In the next experiments, we investigated whether AVP/dDAVP stimulation of mTAL leads to the tubular secretion of nucleotides. The biosensor cells were positioned in front of the tubular outflow during the entire course of the experiment.

Fig. 2. Biosensor approach adapted to detect nucleotides in the tubular outflow of isolated, perfused renal tubules. **A**: concentration-response curve of ATP-induced $[Ca^{2+}]_i$ increase in hP2Y₂-132-1N1 cells. **B**: summary of all nucleotide-induced concentration-response curves in hP2Y₂-132-1N1 cells. Note that non-P2 receptor-expressing 132-1N1 cells showed no $[Ca^{2+}]_i$ increase in ATP and UTP. **C**: a fast agonist application set-up to administer very short pulses of nucleotides to biosensor cells. **D**: an original $[Ca^{2+}]_i$ recording of a biosensor cell receiving 3 short (1 s) and one longer (~50 s) UTP (1 μ M) stimulation.



Only experiments which met the following criteria were included in the study. 1) UTP applied to the bath at the end of the experiments elicited brisk $[Ca^{2+}]_i$ increases in tubular and biosensor cells. 2) The baseline $[Ca^{2+}]_i$ of the biosensor cells was silent for several minutes before the experiment was initiated. In about 30% of the experiments, the biosensor cells continued to display ongoing $[Ca^{2+}]_i$ oscillatory behavior even after they had been positioned into the outflow for 10–20 min. 3) The positions of the biosensor cells and the tubule did not change during the course of the experiment. 4) The luminal fluid flow marker (fura 2, potassium salt) surrounded the biosensor cells before and after the experiment. 5) The tubule responded to 10 nM AVP/dDAVP. The latter was the case in almost all experiments (>90% of all experiments). Figure 4A shows one typical recording of the AVP effect (10 nM) on tubular and biosensor $[Ca^{2+}]_i$. AVP increased mTAL $[Ca^{2+}]_i$ in an oscillatory fashion, in agreement with the experiments shown in Fig. 1. The ordinate in Fig. 4 is scaled to accommodate the large biosensor $[Ca^{2+}]_i$ signal. After a delay of ~120 s, a single, large transient $[Ca^{2+}]_i$ increase could be detected in the biosensor cells (see also Supplementary video 2). In the AVP series, 11 experiments met all inclusion criteria and in 7 experiments of these at least 1 biosensor $[Ca^{2+}]_i$ increase was observed. In these 7 experiments, altogether 20 transient $[Ca^{2+}]_i$ increases were recorded in the biosensor and analyzed further. Their mean and single relative fluo 4 fluorescence increases are summarized in Fig. 4C. As stated above, these $[Ca^{2+}]_i$ bursts were never observed in the 5-min period before addition of the hormone. In a second series, we investigated the effect of the V2 receptor agonist dDAVP. In this series, 11 experiments met the inclusion criteria and in 8 experiments dDAVP induced one or more biosensor $[Ca^{2+}]_i$ increments, as shown in Fig. 4B. In these 8 experiments, a total of 16

$[Ca^{2+}]_i$ transients were observed. The summary of all dDAVP-triggered biosensor $[Ca^{2+}]_i$ elevations is given in Fig. 4C. In the entire AVP/dDAVP series, the delay between the addition of the hormone and the first $[Ca^{2+}]_i$ increase was 99 ± 39 s ($n = 15$). Plotting all AVP/dDAVP-induced biosensor $[Ca^{2+}]_i$ increases into a histogram as a function of time after hormone stimulation (Fig. 4D) showed an equal distribution of $[Ca^{2+}]_i$ bursts over a 5-min period. These data indicate that the AVP/dDAVP-induced tubular $[Ca^{2+}]_i$ increases roughly coincide with the $[Ca^{2+}]_i$ increases observed in the biosensor cells (Fig. 3). By plotting the mean AVP/dDAVP-induced $[Ca^{2+}]_i$ burst amplitudes of the biosensor cells into the combined ATP/UTP concentration response curve (derived from Fig. 2B), the approximated intratubular peak nucleotide concentration can be estimated to be in the range of 0.2–0.3 μ M. These data make us conclude that vasopressin receptor stimulation by AVP and dDAVP in mouse mTAL triggers the tubular secretion of nucleotides which can be detected with ATP/UTP-sensing biosensor cells. This stimulated secretion appears to occur in bursts.

AVP triggers nucleotide secretion from perfused CCD. Next, we investigated whether AVP stimulates tubular secretion of nucleotides from the CCD. The CCD from different species is known to respond to nanomolar concentrations of AVP with a significant increase in $[Ca^{2+}]_i$. As shown in Fig. 5A, this is also the case for mouse CCD, where 10 nM AVP produced a slow, smooth increase in $[Ca^{2+}]_i$. We found three characteristics that differ in the AVP-induced $[Ca^{2+}]_i$ response of CCD compared with mTAL. At 10 nM AVP, localized $[Ca^{2+}]_i$ increases were rarely observed and the increase was homogenous and global over the entire CCD. Lower concentrations of AVP were not investigated. Second, the period from the start of the stimula-

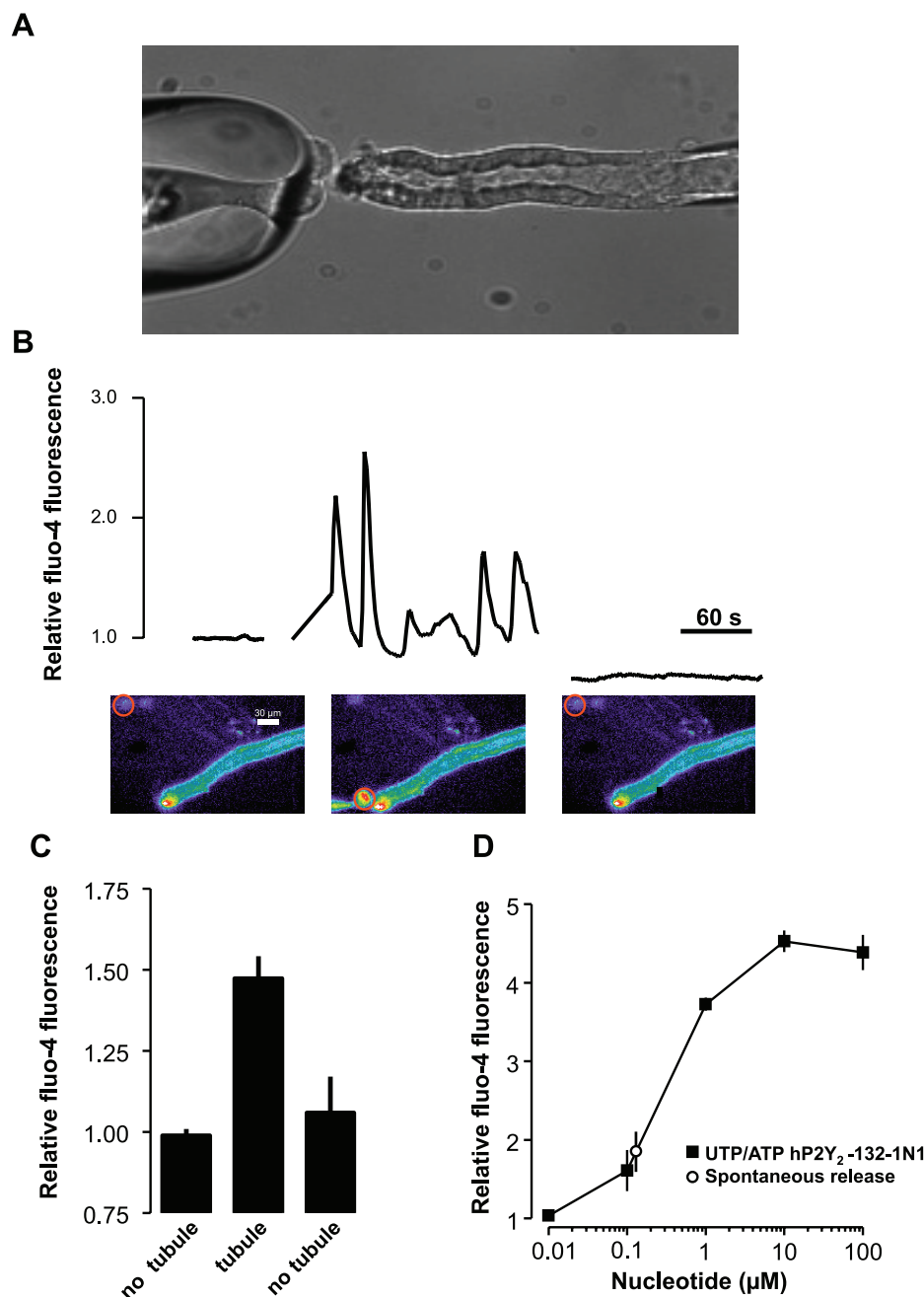


Fig. 3. Detection of biosensor cell $[Ca^{2+}]_i$ increase when positioned directly in the tubular outflow of an isolated, perfused mTAL. *A*: transmission image of a perfused medullary thick ascending limb with the open tubule end pointing directly toward 2 single hP2Y₂-132-1N1 cells attached to a fire-polished glass pipette. *B*: original recording of biosensor $[Ca^{2+}]_i$ increases during 3 time periods: biosensor cell away from tubule (*left trace* and inset; biosensor cell is in the *top left corner* encircled in red); biosensor cell directly in front of the tubular outflow (*middle trace* and inset); and cell moving away from the tubular outflow (*right trace* and inset). *C*: summary. Mean relative fluo 4 fluorescence increase is shown during the 3-min period of the biosensor cells placed in front of the tubular outflow. *D*: quantification of the intraluminal nucleotide concentration.

tion to the maximal response was dramatically longer and amounted to ~ 100 s (see Figs. 1*B* and 5, *A–C*). Finally, the AVP-induced maximal $[Ca^{2+}]_i$ transients were significantly larger in the CCD compared with mTAL (see Figs. 1*B* and 5*B*). We used the same inclusion criteria for CCDs as described for mTAL. In Fig. 5*C*, a typical recording shows the effect on AVP (10 nM) on tubular and biosensor $[Ca^{2+}]_i$. AVP increased CCD $[Ca^{2+}]_i$ similar to that depicted in Fig. 5, *A* and *B*. After a delay of ~ 60 s, a transient $[Ca^{2+}]_i$ increase could be detected in the biosensor cells which is followed by a second one immediately after the removal of basolateral AVP (see Supplementary video 3). In the AVP series of experiments, seven tubules met all inclusion criteria and in all seven tubules at least one biosensor $[Ca^{2+}]_i$ increase was observed. In these

7 experiments, altogether 11 transient $[Ca^{2+}]_i$ increases were recorded and further analyzed. Their mean and single relative fluo 4 fluorescence increases are summarized in Fig. 5*D*. During AVP stimulation (~ 3 min), one to three $[Ca^{2+}]_i$ bursts were detected. These $[Ca^{2+}]_i$ bursts were never observed in the periods (5 min) before addition of the hormone. In the entire AVP CCD series, the delay between the addition of the hormone and the first biosensor $[Ca^{2+}]_i$ increase amounted to 105 ± 47 s ($n = 7$), and they all coincided with the increase in CCD $[Ca^{2+}]_i$. By plotting the mean AVP-induced $[Ca^{2+}]_i$ burst amplitudes into the combined ATP/UTP concentration-response curve (derived from Fig. 2*B*), the approximated intratubular CCD peak nucleotide concentration can be estimated to be $0.3 \mu\text{M}$. Therefore, vasopressin receptor stimulation by

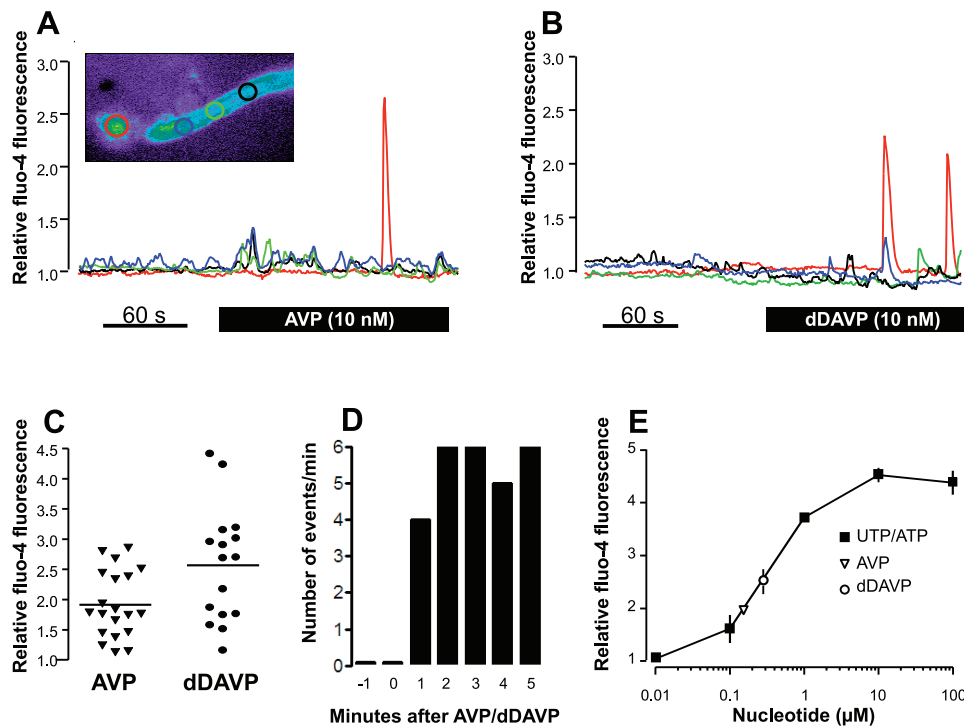


Fig. 4. AVP- and dDAVP-induced nucleotide secretion from isolated, perfused mouse mTAL. *A*: original fluo 4 $[Ca^{2+}]_i$ recording of perfused medullary thick ascending limb in parallel with a nucleotide biosensor cell positioned in its outflow. Tubular $[Ca^{2+}]_i$ is measured in 3 regions of interest (black, blue, and green traces), and biosensor $[Ca^{2+}]_i$ is shown as the red trace. After addition of 10 nM AVP, the tubular $[Ca^{2+}]_i$ begins to oscillate similar to that described in Fig. 3. After a delay of 100 s, a single, large increase in $[Ca^{2+}]_i$ is measured in the biosensor cells. *B*: the same type of experiment is shown, and now dDAVP is applied. The biosensor $[Ca^{2+}]_i$ signal is shown in red. After the addition of dDAVP (10 nM), 2 $[Ca^{2+}]_i$ bursts are detected in the biosensor cells. The first burst occurred after ~ 90 s. *C*: summary of the magnitude of all AVP- and dDAVP-induced biosensor $[Ca^{2+}]_i$ bursts as measured from 7 and 8 mTAL tubules, respectively. *D*: histogram of all measured $[Ca^{2+}]_i$ bursts induced after the addition of either AVP and dDAVP. During a recording period of 5 min after the addition of the agonist, 27 $[Ca^{2+}]_i$ bursts were detected and were found to be equally distributed within the five 1-min observation intervals. *E*: quantification of the AVP/dDAVP-induced peak nucleotide concentrations with the biosensor approach. AVP and dDAVP induced apparent peak nucleotide concentrations of ~ 0.2 and ~ 0.3 μM , respectively.

AVP in mouse CCD triggers the tubular secretion of nucleotides which can be detected with ATP/UTP-sensing biosensor cells. Also in the CCD, this stimulated secretion appears to occur in bursts. These results indicate that AVP-induced nucleotide secretion is a phenomenon present in both the mTAL and the CCD.

DISCUSSION

Renal tubular transport is fundamental for body salt and water homeostasis. Systemic and local hormonal signals regulate tubular transport and thus define the final composition of the excreted urine and hereby body volume and electrolyte status. Extracellular nucleotides modulate epithelial functions via P2 receptor activation, including renal ion and water transport (17, 34, 37). Extensive taxonomic work has defined P2 receptors to be expressed along the entire renal tubular system, both on the luminal and basolateral sides (17, 34, 36). Significant evidence underscores an important role of the G protein-coupled P2Y₂ receptor. Acute stimulation of this receptor reduces distal tubular, ENaC-mediated Na⁺ absorption and AQP2-conducted water transport (13, 16, 27, 32, 35). Recent indirect evidence supports that this receptor is also involved in the regulation of inner medullary urea transport (44). In mice, global lack of the P2Y₂ receptor is associated with hypertension (30). The hypertension is most likely caused by volume expansion assumed to result from removal of tonal

inhibition of transport along the renal tubule in the absence of the P2Y₂ receptor (31). This finding does not only underscore the importance for P2 receptors in maintaining normal renal function, it also implies that extracellular nucleotides are continually present as local signaling molecules in the kidney. Increasing evidence supports that the epithelial cells themselves are the source of extracellular ATP (15, 29). However, the mechanism and regulation of renal tubular ATP release are still poorly understood (29). Nonetheless, a number of recent observations present valuable progress in this field. Similar to other epithelial cells, it has become clear that renal tubular cells release nucleotides spontaneously (6, 25, 27, 38). This can be measured directly as the spontaneous appearance of ATP in the surrounding fluid (6, 38) and is also reflected in slow, spontaneous $[Ca^{2+}]_i$ oscillations (6). Here, we confirm that isolated, perfused mTAL segments display spontaneous nucleotide release, which in this study was detected in the luminal fluid compartment by hP2Y₂ receptor-expressing 132-1N1 cells placed in the outflow of an isolated, perfused mouse mTAL. The biosensors showed significant increments in $[Ca^{2+}]_i$ when placed in the outflow of the mTAL, which was not seen in cells not expressing the P2 receptors. The mean amplitude of these $[Ca^{2+}]_i$ transients correspond to a peak nucleotide concentration in the tubular outflow of ~ 0.2 μM . This value is almost identical with the level detected by micropuncture in the only other study quantifying intraluminal ATP concentrations in rat

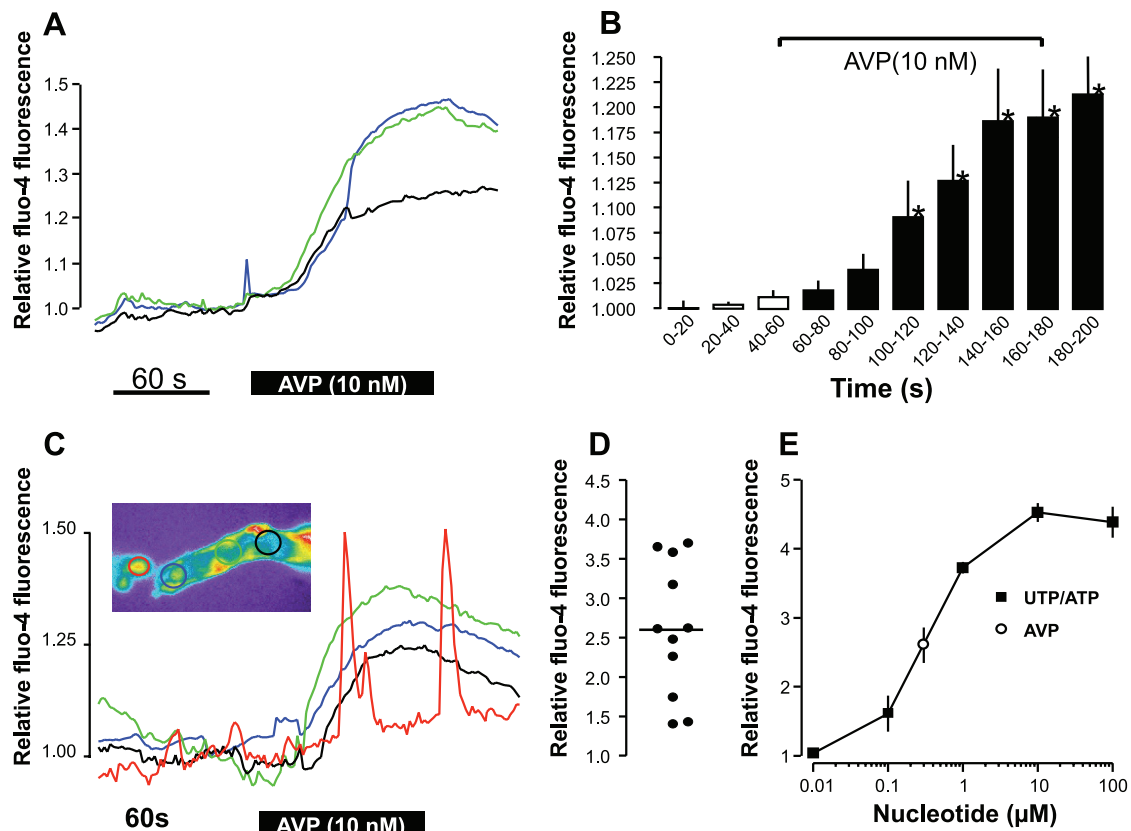


Fig. 5. AVP-induced nucleotide secretion from isolated, perfused mouse cortical collecting duct (CCD). **A:** original recording of an AVP (10 nM)-induced $[Ca^{2+}]_i$ increase in mouse CCD as recorded over 3 circular regions of interest, as indicated in fluorescence image in **C**. A smooth and slow increase was observed, with a peak $[Ca^{2+}]_i$ elevation being measured after ~ 100 s. **B:** summary of AVP-induced $[Ca^{2+}]_i$ increase in mouse CCD. **C:** original fluo 4 $[Ca^{2+}]_i$ recording of perfused CCD in parallel with a nucleotide biosensor cell positioned in its outflow. Tubular $[Ca^{2+}]_i$ is measured in 3 regions of interest (black, blue, and green traces) and biosensor $[Ca^{2+}]_i$ is shown as the red trace. After addition of 10 nM AVP, the tubular $[Ca^{2+}]_i$ increased. After a delay of ~ 60 s, 2 large increases in $[Ca^{2+}]_i$ were measured in the biosensor cells. **D:** summary of the magnitude of all AVP-induced biosensor $[Ca^{2+}]_i$ bursts as measured from 7 CCD tubules. **E:** quantification of the AVP-induced peak nucleotide concentrations with the biosensor approach. AVP induced apparent peak nucleotide concentrations of ~ 0.3 μ M.

proximal tubules (38). Frequently, we observed an oscillating $[Ca^{2+}]_i$ signal in the biosensor cells. This is commonly found in $G_{\alpha q}$ -coupled receptors when stimulated with low agonist concentrations (2). In the experiments shown in Fig. 2B, a low concentration of 0.1 μ M ATP did not induce prominent $[Ca^{2+}]_i$ oscillations. The oscillatory $[Ca^{2+}]_i$ signals in the biosensor cells may therefore reflect rapidly changing nucleotide concentrations in the luminal fluid. Other more refined methods using e.g., nondesensitizing nucleotide outside-out patches (e.g., with $P2X_2$ receptors) could be helpful (9). Using $hP2Y_2$ receptors in the biosensor cells allows detection of both, UTP and ATP, which is relevant as the renal tubules are able to respond to both of these nucleotides. This approach precludes from distinguishing which of the two nucleotides is released.

Recent studies have shown that stimulation of membrane receptors, which triggers an increase in $[Ca^{2+}]_i$, also provokes a parallel release of ATP. Most of these receptors are GPCRs, which couple to an increase in $[Ca^{2+}]_i$ via the generation of IP_3 . The use of direct stimulation of G proteins with GTP- γ -S or the G protein activator compound 48/80 strongly suggests that G protein activation is necessary for agonist-stimulated ATP release (8, 24). In addition, most of these studies clearly indicated that the intracellular Ca^{2+} chelator BAPTA strongly

or completely inhibited the agonist-induced ATP release (8, 11, 18, 19). Receptor-independent $[Ca^{2+}]_i$ elevations with, e.g., ionomycin or A23187 are apparently poor agonists to release nucleotides (3, 8, 23). Additional evidence indicates that inhibition of phospholipase C with U73122 can block agonist-triggered ATP release (8, 19). Thus it appears likely that GPCR agonists in general can trigger ATP release from many different cell types. In this study, we present evidence which indicates that AVP is able to trigger the secretion of nucleotides in renal tubules. Three different experimental series were conducted, two in the mTAL and one in the CCD. During the experiments, the tubular and the biosensor $[Ca^{2+}]_i$ was measured in parallel, and we found a reasonable temporal correlation between the tubular $[Ca^{2+}]_i$ signal and that induced in the biosensor cells. In both tubular segments, we found a similar pattern of AVP-induced nucleotide secretion. It appeared to occur in bursts, which is then washed along with the tubular fluid to be detected as a transient $[Ca^{2+}]_i$ increase in the biosensor cells. Our findings are supported by recent results that show significantly reduced urinary ATP concentration in mice after treatment with a V2 receptor antagonist (30).

Several studies have investigated AVP-induced $[Ca^{2+}]_i$ signaling in different thick ascending limb preparations and showed a transient increase in $[Ca^{2+}]_i$ where 10 nM AVP was

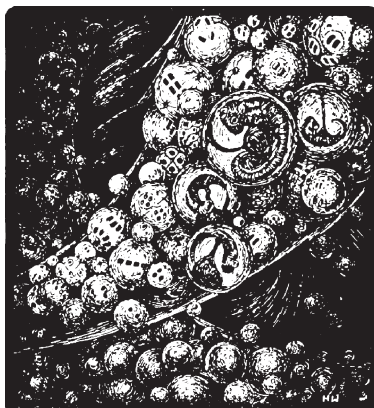
found to induce a maximal effect (4, 12). Our study provides a novel perspective of this phenomenon, namely, that the AVP-induced $[Ca^{2+}]_i$ increase is actually composed of numerous randomly occurring, locally confined $[Ca^{2+}]_i$ increases. When this phenomenon is measured in the entire tubule, the single effects likely meld to provide a smooth, seemingly global $[Ca^{2+}]_i$ transient. Rodent mTAL abundantly and predominantly expresses V2 receptors (12, 21), and our data with dDAVP suggest that the $[Ca^{2+}]_i$ effect is mediated via this receptor. This is supported from rat data, which indicate that a V2 antagonist completely abrogated the AVP-induced $[Ca^{2+}]_i$ increase (12). Some care must be taken with this conclusion as the human V1b receptor can be activated with nanomolar concentrations of dDAVP (33). Noteworthy, in rabbit cTAL AVP increases $[Ca^{2+}]_i$ via activation of the V1 receptor (22). Thus our current data do not allow us to firmly conclude which vasopressin receptor is activated and responsible for the AVP/dDAVP-induced effect in mouse mTAL. In this context, it is interesting to appreciate the rather atypical character of the AVP-induced $[Ca^{2+}]_i$ elevation in both the mTAL and CCD. GPCR stimulation commonly increases $[Ca^{2+}]_i$ shortly after agonist addition, within the first very few seconds. In both tubular segments, the AVP-induced $[Ca^{2+}]_i$ transients/oscillations increase very slowly. In the mTAL, an integrated $[Ca^{2+}]_i$ maximum is reached only between 20 and 40 s, and in the CCD this is even longer and occurs after ~ 100 s. In Supplementary Fig. 1, these pronounced kinetic differences are shown for basolateral ATP and AVP in mouse CCD. These results could argue against a direct effect of AVP on a vasopressin receptor to induce IP_3 -dependent increases in $[Ca^{2+}]_i$ and may indicate that the AVP-induced tubular $[Ca^{2+}]_i$ transient occurs secondarily to other preceding but currently unknown processes. In light of our findings, one may speculate that the AVP-induced tubular $[Ca^{2+}]_i$ increase could encompass a paracrine purinergic component.

In conjunction, we envisage the following integrative perspective of these results. Paracrine nucleotides are prominent inhibitory modulators of renal tubular transport. This is best described for ENaC-mediated Na^+ and AQP-2-mediated water absorption in the cortical and inner medullary collecting duct. The agonist AVP predominantly activates transport in the collecting duct and the thick ascending limb. Thus these two systems, ATP and AVP, apparently oppose each other's transport effects. We know that AVP regulates the activation of transport and speculate that it in parallel, possibly at higher concentrations, costimulates the release of nucleotides which would oppose transport activation via a local inhibitory feedback signal.

REFERENCES

- Bailey MA, Imbert-Teboul M, Turner C, Marsy S, Srai K, Burnstock G, Unwin RJ. Axial distribution and characterization of basolateral P2Y receptors along the rat renal tubule. *Kidney Int* 58: 1893–1901, 2000.
- Berridge MJ. Annual Review Prize Lecture: elementary and global aspects of calcium signalling. *J Physiol* 499: 291–306, 1997.
- Boudreau F, Grygorczyk R. Cell swelling-induced ATP release is tightly dependent on intracellular calcium elevations. *J Physiol* 561: 499–513, 2004.
- Dai LJ, Quamme GA. Hormone-mediated Ca^{2+} transients in isolated renal cortical thick ascending limb cells. *Pflügers Arch* 427: 1–8, 1994.
- Ecelbarger CA, Chou CL, Lolait SJ, Knepper MA, DiGiovanni SR. Evidence for dual signaling pathways for V2 vasopressin receptor in rat inner medullary collecting duct. *Am J Physiol Renal Fluid Electrolyte Physiol* 270: F623–F633, 1996.
- Geyti CS, Odgaard E, Overgaard MT, Jensen ME, Leipziger J, Praetorius HA. Slow spontaneous $[Ca^{2+}]_i$ oscillations reflect nucleotide release from renal epithelia. *Pflügers Arch* 455: 1105–1117, 2008.
- Greger R, Hampel W. A modified system for in vitro perfusion of isolated renal tubules. *Pflügers Arch* 389: 175–176, 1981.
- Gruenhagen JA, Yeung ES. Investigation of G protein-initiated, Ca^{2+} -dependent release of ATP from endothelial cells. *Biochim Biophys Acta* 1693: 135–146, 2004.
- Hayashi S, Hazama A, Dutta AK, Sabirov RZ, Okada Y. Detecting ATP release by a biosensor method. *Sci STKE* 2004: 114, 2004.
- Jensen ME, Odgaard E, Christensen MH, Praetorius HA, Leipziger J. Flow-induced $[Ca^{2+}]_i$ increase depends on nucleotide release and subsequent purinergic signaling in the intact nephron. *J Am Soc Nephrol* 18: 2062–2070, 2007.
- Joseph SM, Buchakjian MR, Dwyer GR. Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *J Biol Chem* 278: 23331–23342, 2003.
- Jung KY, Endou H. A novel vasopressin receptor in rat early proximal tubule. *Biochem Biophys Res Commun* 180: 131–137, 1991.
- Kishore BK, Chou CL, Knepper MA. Extracellular nucleotide receptor inhibits AVP-stimulated water permeability in inner medullary collecting duct. *Am J Physiol Renal Fluid Electrolyte Physiol* 269: F863–F869, 1995.
- Knepper MA, Nielsen S, Chou CL, DiGiovanni SR. Mechanism of vasopressin action in the renal collecting duct. *Semin Nephrol* 14: 302–321, 1994.
- Lazarowski ER, Boucher RC, Harden TK. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* 64: 785–795, 2003.
- Lehrmann H, Thomas J, Kim SJ, Jacobi C, Leipziger J. Luminal P2Y₂ receptor-mediated inhibition of Na^+ absorption in isolated perfused mouse CCD. *J Am Soc Nephrol* 13: 10–18, 2002.
- Leipziger J. Control of epithelial transport via luminal P2 receptors. *Am J Physiol Renal Physiol* 284: F419–F432, 2003.
- Liu GJ, Kalous A, Werry EL, Bennett MR. Purine release from spinal cord microglia after elevation of calcium by glutamate. *Mol Pharmacol* 70: 851–859, 2006.
- Liu GJ, Werry EL, Bennett MR. Secretion of ATP from Schwann cells in response to uridine triphosphate. *Eur J Neurosci* 21: 151–160, 2005.
- Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. *Pharmacol Rev* 53: 245–282, 2001.
- Mutig K, Paliege A, Kahl T, Jons T, Müller-Esterl W, Bachmann S. Vasopressin V2 receptor expression along rat, mouse, and human renal epithelia with focus on TAL. *Am J Physiol Renal Physiol* 293: F1166–F1177, 2007.
- Nitschke R, Fröbe U, Greger R. Antidiuretic hormone acts via V₁ receptors on intracellular calcium in the isolated perfused rabbit cortical thick ascending limb. *Pflügers Arch* 417: 622–632, 1991.
- Okada S, Paradiso AM, Lazarowski ER, Boucher RC. A calcium-dependent pathway in swelling-induced ATP release from airway epithelial cells. *J Physiol* 567: C53, 2006.
- Osipchuk Y, Cahalan M. Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. *Nature* 359: 241–244, 1992.
- Ostrom RS, Gregorian C, Insel PA. Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. *J Biol Chem* 275: 11735–11739, 2000.
- Parr CE, Sullivan DM, Paradiso AM, Lazarowski ER, Burch LH, Olsen JC, Weisman GA, Boucher RC, Turner JT. Cloning and expression of a human P2U nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc Natl Acad Sci USA* 91: 3275–3279, 1994.
- Pochynuk O, Bugaj V, Rieg T, Insel PA, Mironova E, Vallon V, Stockand JD. Paracrine regulation of the epithelial Na^+ channel in the mammalian collecting duct by purinergic P2Y₂ receptor tone. *J Biol Chem* 283: 36599–36607, 2008.
- Praetorius HA, Leipziger J. Fluid flow sensing and triggered nucleotide release in epithelia. *J Physiol* 586: 2669, 2008.
- Praetorius HA, Leipziger J. ATP release from non-excitable cells. *Purinergic Signal*. In press.
- Rieg T, Bundey RA, Chen Y, Deschenes G, Junger WG, Insel PA, Vallon V. Mice lacking P2Y₂ receptors have salt-insensitive hypertension and facilitated renal Na^+ and water reabsorption. *FASEB J* 13, 3717–3726, 2007.

31. **Rieg T, Vallon V.** ATP and adenosine in the local regulation of water transport and homeostasis by the kidney. *Am J Physiol Regul Integr Comp Physiol* 296: R419–R427, 2009.
32. **Rouse D, Leite M, Suki WN.** ATP inhibits the hydrostatic effect of AVP in rabbit CCT: evidence for a nucleotide P2U receptor. *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F289–F295, 1994.
33. **Saito M, Tahara A, Sugimoto T.** 1-Desamino-8-D-arginine vasopressin (DDAVP) as an agonist on V1b vasopressin receptor. *Biochem Pharmacol* 53: 1711–1717, 1997.
34. **Schwiebert EM, Kishore BK.** Extracellular nucleotide signaling along the renal epithelium. *Am J Physiol Renal Physiol* 280: F945–F963, 2001.
35. **Thomas J, Deetjen P, Ko WH, Jacobi C, Leipziger J.** P2Y₂ receptor-mediated inhibition of amiloride-sensitive short circuit current in M-1 mouse cortical collecting duct cells. *J Membr Biol* 183: 115–124, 2001.
36. **Unwin RJ, Bailey MA, Burnstock G.** Purinergic signaling along the renal tubule: the current state of play. *News Physiol Sci* 18: 237–241, 2003.
37. **Vallon V.** P2 receptors in the regulation of renal transport mechanisms. *Am J Physiol Renal Physiol* 294: F10–F27, 2008.
38. **Vekaria RM, Unwin RJ, Shirley DG.** Intraluminal ATP concentrations in rat renal tubules. *J Am Soc Nephrol* 17: 1841–1847, 2006.
39. **Weiner ID, Hamm LL.** Use of fluorescent dye BCECF to measure intracellular pH in cortical collecting tubule. *Am J Physiol Renal Fluid Electrolyte Physiol* 256: F957–F964, 1989.
40. **Woo K, Dutta AK, Patel V, Kresge C, Feranchak AP.** Fluid-flow induces mechanosensitive ATP release, calcium signaling, and Cl[−] transport in biliary epithelial cells through a PKC ζ -dependent pathway. *J Physiol* 586: 2779–2798, 2008.
41. **Wright PA, Burg MB, Knepper MA.** Microdissection of kidney tubule segments. *Methods Enzymol* 191: 226–231, 1990.
42. **Xu C, Shmukler BE, Nishimura K, Kaczmarek E, Rossetti S, Harris PC, Wandering-Ness A, Bacallao RL, Alper SL.** Attenuated, flow-induced ATP release contributes to absence of flow-sensitive, purinergic Ca²⁺ signaling in human ADPKD cyst epithelial cells. *Am J Physiol Renal Physiol* 296: F1464–F1476, 2009.
43. **Yip KP.** Coupling of vasopressin-induced intracellular Ca²⁺ mobilization and apical exocytosis in perfused rat kidney collecting duct. *J Physiol* 538: 891–899, 2002.
44. **Zhang Y, Sands JM, Kohan DE, Nelson RD, Martin CF, Carlson NG, Kamerath CD, Ge Y, Klein JD, Kishore BK.** Potential role of purinergic signaling in urinary concentration in inner medulla: insights from P2Y₂ receptor gene knockout mice. *Am J Physiol Renal Physiol* 295: F1715–F1724, 2008.



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