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Effects of luminal flow and nucleotides on $[Ca^{2+}]_i$ in rabbit cortical collecting duct

CRAIG B. WODA,[1] MAURILO LEITE, JR.,[2] RAJEEV ROHATGI,[3] AND LISA M. SATLIN[1,3]
Departments of [1]Pediatrics and [3]Medicine, Mount Sinai School of Medicine, New York, New York 10029-6574; and [2]Department of Medicine, Universidade Federal do Rio de Janeiro, Hospital Universitario Clementino Fraga Filho, 21949-900 Rio de Janeiro, Brazil

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Woda, Craig B., Maurilo Leite, Jr., Rajeev Rohatgi, and Lisa M. Satlin. Effects of luminal flow and nucleotides on $[Ca^{2+}]_i$ in rabbit cortical collecting duct. *Am J Physiol Renal Physiol* 283: F437–F446, 2002. First published May 14, 2002; 10.1152/ajprenal.00316.2001.—Nucleotide binding to purinergic P2 receptors contributes to the regulation of a variety of physiological functions in renal epithelial cells. Whereas P2 receptors have been functionally identified at the basolateral membrane of the cortical collecting duct (CCD), a final regulatory site of urinary Na^+, K^+, and acid-base excretion, controversy exists as to whether apical purinoceptors exist in this segment. Nor has the distribution of receptor subtypes present on the unique cell populations that constitute Ca^{2+} the CCD been established. To examine this, we measured nucleotide-induced changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura 2-loaded rabbit CCDs microperfused in vitro. Resting $[Ca^{2+}]_i$ did not differ between principal and intercalated cells, averaging ~120 nM. An acute increase in tubular fluid flow rate, associated with a 20% increase in tubular diameter, led to increases in $[Ca^{2+}]_i$ in both cell types. Luminal perfusion of 100 μM UTP or ATP-γ-S, in the absence of change in flow rate, caused a rapid and transient approximately fourfold increase in $[Ca^{2+}]_i$ in both cell types ($P < 0.05$). Luminal suramin, a nonspecific P2 receptor antagonist, blocked the nucleotide- but not flow-induced $[Ca^{2+}]_i$ transients. Luminal perfusion with a P2X (α,β-methylene-ATP), $P2X_7$ (benzoyl-benzoyl-ATP), $P2Y_1$ (2-methylthio-ATP), or $P2Y_4$/$P2Y_6$ (UDP) receptor agonist had no effect on $[Ca^{2+}]_i$. The nucleotide-induced $[Ca^{2+}]_i$ transients were inhibited by the inositol-1,4,5-triphosphate receptor blocker 2-aminoethoxydiphenyl borate, thapsigargin, which depletes internal Ca^{2+} stores, luminal perfusion with a Ca^{2+}-free perfusate, or the L-type Ca^{2+} channel blocker nifedipine. These results suggest that luminal nucleotides activate apical $P2Y_2$ receptors in the CCD via pathways that require both internal Ca^{2+} mobilization and extracellular Ca^{2+} entry. The flow-induced rise in $[Ca^{2+}]_i$ is apparently not mediated by apical P2 purinergic receptor signaling.

microperfusion; fura 2; purinergic receptor; principal cell; intercalated cell; intracellular calcium concentration

CUMULATIVE EVIDENCE INDICATES that ion transport across epithelia is regulated by binding of extracellular nucleotides to purinergic receptors present on cell surfaces. Two main groups of purinergic receptors have been identified based on their pharmacological properties. P2 purinergic receptors are activated by ATP, ADP, UTP, and UDP, whereas P1 purinoceptors respond preferentially to AMP and adenosine, the breakdown products of ATP hydrolysis (14). Within the P2 purinoceptor family, P2X receptors are intrinsic ion channels that mediate depolarization and influx of Ca^{2+}, whereas P2Y receptors are coupled to heterotrimeric G proteins, phospholipases, and phosphoinositol signaling pathways (14, 46, 57). Specific subtypes of P2X or P2Y receptors can be identified based on their response to specific nucleotide agonists (14, 46, 57).

The P2Y receptor family is comprised of at least five distinct molecular subtypes ($P2Y_{1,2,4,6,11}$; reviewed in Ref. 57). Binding of ATP to the G protein-coupled P2Y receptor activates phospholipase C (PLC), leading to inositol-1,4,5-triphosphate (IP_3) production and mobilization of internal Ca^{2+} stores (14, 46, 57). P2Y receptor activation has also been shown to stimulate production of diacylglycerol and protein kinase C, modulate adenylate cyclase activity and cAMP production, and stimulate the formation and release of prostaglandins (1, 14, 30, 46, 57, 68). P2X receptors, comprising at least seven subtypes ($P2X_{1-7}$) (46, 57), form Ca^{2+}-permeable nonselective cation channels that, on activation, allow for Ca^{2+} entry from the extracellular milieu into the cell (6, 60).

The cortical collecting duct (CCD) of the mammalian nephron contributes to the final renal regulation of Na^+, K^+, acid-base, and water homeostasis. The CCD is a heterogeneous epithelium comprising two morphologically and functionally distinct cell types. Whereas principal cells reabsorb Na^+ and water (in the presence of vasopressin) and secrete K^+, intercalated cells transport acid-base and can, under certain conditions, absorb K^+ (10, 29, 52, 56, 59). Although these cells reside directly adjacent to each other within the CCD, they are considered not to be coupled, maintaining different resting intracellular pH (54).

Functional studies of CCD cells grown in culture (30, 62), isolated tubules (9, 34), and established cell lines

Address for reprint requests and other correspondence: L. M. Satlin, Box 1664, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029-6574 (E-mail: lisa.satlin@mssm.edu).

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expressing properties typical of CCD principal cells (36, 41) provide evidence for the presence of P2Y receptors in this segment. However, few studies have been directed at delineating the polarity of these receptors to the apical or basolateral membranes. A basolateral localization of $P2Y_2$ (previously known as P2U) receptors has been supported by the observations that peritubular ATP and UTP cause a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in nonperfused rat CCDs (9) and inhibit the hydrosmotic action of vasopressin in microperfused rabbit CCDs (49). Although apical and basolateral $P2Y_2$ receptors have been identified in primary cultures of rabbit CCD cells (30), Deetjen et al. (12) found no functional apical P2 receptors in microperfused rabbit CCDs. Recently, Kishore et al. (28) detected $P2Y_2$ receptor mRNA in microdissected rat CCDs. Although immunoreactive $P2Y_2$ receptor was identified along the apical, and to a lesser extent basolateral, membranes of collecting duct principal cells in the inner medulla, the localization of the protein in the CCD was not explored (28).

The purpose of the present study was to *1*) determine whether functional P2 receptors are present on the apical surfaces of rabbit CCD cells and, if so, *2*) identify the distribution of receptor subtypes present on the unique cell populations that constitute the CCD. On the basis of our results, we also sought to identify the source of Ca^{2+} giving rise to the nucleotide-induced $[Ca^{2+}]_i$ transients. To accomplish these aims, we used the Ca^{2+}-sensitive fluorescent dye fura 2 to measure changes in principal and intercalated cell $[Ca^{2+}]_i$ in isolated CCDs microperfused with nucleotide analogs. As an incidental finding, we noted that epithelial stretch induced by rapid increases in tubular fluid flow rate led to $[Ca^{2+}]_i$ transients in the CCD. The role of apical P2 purinergic signaling in this response was investigated.

MATERIALS AND METHODS

Animals. Adult female New Zealand White rabbits were obtained from Covance (Denver, PA) and housed in the Mount Sinai School of Medicine animal care facility. The animals were fed standard rabbit chow and given free access to food and water. Animals were killed by intraperitoneal injection of a lethal dose of pentobarbital sodium (100 mg/kg). All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academy Press, 1996).

In vitro microperfusion. The kidneys were removed via a midline incision, sliced into 2-mm coronal sections, and single tubules were dissected freehand in cold (4°C) dissection solution containing (in mM) 145 NaCl, 2.5 K_2HPO_4, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 4.0 Na lactate, 1.0 Na_3 citrate, 6.0 L-alanine, and 5.5 D-glucose, pH 7.4, 290 ± 2 mosmol/kgH_2O (51). A single tubule was studied from each animal.

Each isolated tubule was immediately transferred to a temperature and O_2/CO_2-controlled specimen chamber, assembled with a No. 1 coverslip (Corning) painted with a 1-μl drop of Cell-Tak (Collaborative Biomedical Products, Bedford, MA). The CCD was mounted on concentric glass pipettes, cannulated, and then positioned directly on the Cell-Tak to immobilize the segment for the duration of the experiment. We previously showed that Cell-Tak does not alter the accessibility of the CCD basolateral membrane to the extracellular medium (10, 58). Tubules were perfused and bathed at 37°C with Burg's solution, which resembled the dissection solution except that 25 mM NaCl was replaced by $NaHCO_3$ and the solution was gassed with 95% O_2-5% CO_2 at room temperature to reach a pH of 7.4 (51). In some experiments, CCDs were perfused with Burg's solution prepared without Ca^{2+} (Ca^{2+}-free perfusate). During the 60-min equilibration period and thereafter, the perfusion chamber was continuously suffused with a gas mixture of 95% O_2-5% CO_2 to maintain pH at 7.4 at 37°C. The bathing solution was continuously exchanged at a rate of 10 ml/h using a syringe pump (Razel, Stamford, CT).

Measurement of $[Ca^{2+}]_i$. After equilibration, tubules were loaded with 20 μM of the acetoxymethyl ester of fura 2 (Molecular Probes, Eugene, OR) added to the bath for 20 min. In several experiments, rhodamine-labeled peanut lectin (PNA; Vector Labs, Burlingame, CA) was added to the luminal perfusate for 5 min to identify intercalated cells; rabbit principal cells do not bind PNA (53). With the use of a Nikon Eclipse TE300 inverted epifluorescence microscope linked to a cooled Pentamax charge-coupled device camera (Princeton Instruments) interfaced with a digital imaging system (MetaFluor, Universal Imaging, Westchester, PA), $[Ca^{2+}]_i$ was measured in individually identified fura 2-loaded cells visualized using a Nikon S Fluor ×40 objective (numeric aperture 0.9, working distance 0.3). Autofluorescence was not detectable at the camera gains used. Cells were alternately excited at 340 and 380 nm, and the images were digitized for subsequent analysis. Images were acquired every 2 to 10 s. An intracellular calibration was performed at the conclusion of each experiment according to the technique of Grynkiewicz (24). The 340/380-nm fluorescence ratio was determined initially in the presence of a Ca^{2+}-free bath plus 10 μM EGTA-AM (R_{min}) and then in a 2 mM Ca^{2+} bath containing ionomycin (10 μM; R_{max}). The equation used to calculate experimental values of $[Ca^{2+}]_i$ was $[K_d(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$, where R is the observed ratio of emitted light, K_d is the dissociation constant for fura 2 and Ca^{2+}, assumed to be 224 nM, and S_{f2} and S_{b2} are the fluorescence signals of free and bound dye at 380 nm, respectively (24). Two to six cells were analyzed in each CCD.

Pharmacological classification of P2 receptor subclass. To identify the specific classes of purinergic receptors expressed on the apical membrane of the CCD, fura 2-loaded cells in CCDs perfused at flow rates of ~1–2 $nl \cdot min^{-1} \cdot mm^{-1}$ were monitored for changes in $[Ca^{2+}]_i$ induced by luminal perfusion of the following selective agonists (100 μM): UTP, UDP, ATP and its nonhydrolyzable analog adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), 2-methylthio-ATP, α,β-methylene ATP, and benzoyl-benzoyl-ATP (BzBz-ATP). These ligands are considered to be the highest affinity agonists for their respective receptor subtypes (14, 46, 57). A rank order potency of ATP = UTP > ATP-γ-S > ADP is consistent with the pharmacology of the $P2Y_2$ receptor subtype (14, 46). α,β-Methylene-ATP and BzBz-ATP bind to P2X and $P2X_7$ receptors, whereas 2-methylthio-ATP and UDP are selective for $P2Y_1$ and $P2Y_4$/$P2Y_6$ receptors, respectively (14, 46, 57). Care was taken not to acutely increase tubular fluid flow rate and luminal diameter during exchanges of the luminal perfusate in these experiments (e.g., on addition of luminal nucleotides). In some studies, CCDs were pretreated with luminal suramin (100 μM), a nonspecific P2 receptor antagonist (except for $P2Y_4$) (18, 57), to confirm that the nucleotide-induced $[Ca^{2+}]_i$ transients were mediated by this class of purinergic receptors.

To assess the source of Ca^{2+} contributing to the $[Ca^{2+}]_i$ transient induced by nucleotide stimulation, several CCDs were pretreated with luminal nifedipine (20 μM), an L-type Ca^{2+} channel blocker; 2-aminoethoxydiphenyl borate (2-APB; 10 μM), a cell-permeant inhibitor of the IP_3 receptor (22, 35); or basolateral thapsigargin (100 nM), an irreversible inhibitor of endoplasmic reticulum Ca^{2+}-ATPase that prevents refilling of intracellular Ca^{2+} pools and leads to depletion of internal stores.

Effect of ATP/UTP on gap junctional intercellular communication. Isolated CCDs were split open to expose the apical surfaces of all cells and placed on a coverslip to which Cell-Tak had been previously applied. Microinjection pipettes (Sterile Femtotips II, Eppendorf, Hamburg, Germany) with a tip diameter of 2 μm were filled with Lucifer yellow (20% prepared in normal saline). Randomly identified individual cells were injected with this fluorescent compound using a microinjection system (Micromanipulator 5171, Eppendorf). After injection, the tubule was washed with phosphate-buffered saline. Baseline fluorescence images of the microinjected CCDs were obtained at 5-min intervals for 15 min at room temperature. Thereafter, 100 μM UTP or ATP was added to bathing solution and fluorescence was again monitored for 30 min.

Statistical analysis. Results are expressed as means ± SE; n equals the number of animals, unless otherwise indicated. Significant differences were determined by paired or unpaired t-tests, as appropriate, using the software program SigmaStat (SPSS). Significance was asserted if $P < 0.05$.

RESULTS

Steady-state $[Ca^{2+}]_i$ and response to an increase in flow rate. Intercalated cells, which were differentiated from principal cells in the rabbit CCD by their selective apical binding of rhodamine PNA (Fig. 1), appear more brightly fluorescent under epifluorescence illumination compared with principal cells. Steady-state $[Ca^{2+}]_i$ did not differ between principal and intercalated cells [110 ± 14 vs. 130 ± 17 nM, respectively; P = not significant (NS); $n = 28$].

An acute increase in tubular fluid flow rate in the CCD, sufficient to increase tubular diameter by ~20%, led to a significant ($P < 0.01$) transient increase in $[Ca^{2+}]_i$ in both principal (to 223 ± 22 nM) and intercalated (to 277 ± 33 nM) cells that returned to baseline within 1 min after return to a slower flow rate (Figs. 2 and 3). The flow-induced increase in $[Ca^{2+}]_i$ occurred in most, but not all, CCD cells (Fig. 2). Repetitive acute increases in flow rate (up to 3) from an initial slow flow rate led to parallel increases in $[Ca^{2+}]_i$ (data not shown). However, tubules initially perfused at fast tubular flow rates did not respond to a further increase in flow rate with an incremental increase in $[Ca^{2+}]_i$ (data not shown), suggesting that the cells had already become refractory to flow stimulation. Pretreatment with 100 μM luminal suramin ($n = 4$) did not inhibit the flow-induced increase in $[Ca^{2+}]_i$ in either principal (123 ± 26 to 280 ± 63 nM; $P < 0.05$) or intercalated (111 ± 16 to 262 ± 116 nM; $P < 0.05$) cells, suggesting that apical P2 purinergic signaling does not play a role in this response.

Response of $[Ca^{2+}]_i$ to luminal UTP, ATP, or ATP-γ-S. Luminal perfusion of 5 of 10 CCDs with 100 μM ATP induced a rapid and transient increase followed by a slow return of $[Ca^{2+}]_i$ toward basal values over the next 5 min in intercalated (Fig. 4*A*) and principal (data not shown) cells. Similar increases in $[Ca^{2+}]_i$ were observed in principal (Fig. 4*B*) and intercalated cells (data not shown) in all eight CCDs perfused with 100 μM UTP. In the CCDs that responded to luminal ATP and all segments perfused with UTP, the peak $[Ca^{2+}]_i$ averaged approximately fourfold above baseline in both cell types (Fig. 5). We consider that the inconsistent response of CCDs to luminal ATP reflects the presence of ecto-5′-nucleotidase, an enzyme that catalyzes the breakdown of ATP into adenosine, along the apical cell membrane of intercalated cells in the CCD (32). Luminal perfusion with the poorly hydrolyzable ATP analog ATP-γ-S ($n = 3$) elicited a biphasic increase in $[Ca^{2+}]_i$ similar to that observed in response to UTP. The ATP-γ-S-induced increases in principal and intercalated cell $[Ca^{2+}]_i$ over resting levels were not statistically different from those observed in CCDs that responded to ATP and all segments perfused with UTP (Fig. 5; P = NS). A scatterplot showing the increases in individual principal and intercalated cell $[Ca^{2+}]_i$ elicited by luminal perfusion with UTP or ATP/ATP-γ-S is shown in Fig. 6. Pretreatment of CCDs with luminal suramin blocked the $[Ca^{2+}]_i$ transient induced by luminal perfusion with ATP ($n = 3$) or UTP ($n = 3$) (Fig. 5).

CCDs initially subjected to high tubular flow rates/epithelial stretch did not respond to luminal nucleotide perfusion with an increase in $[Ca^{2+}]_i$ (data not shown). To avoid this apparent flow-induced desensitization,

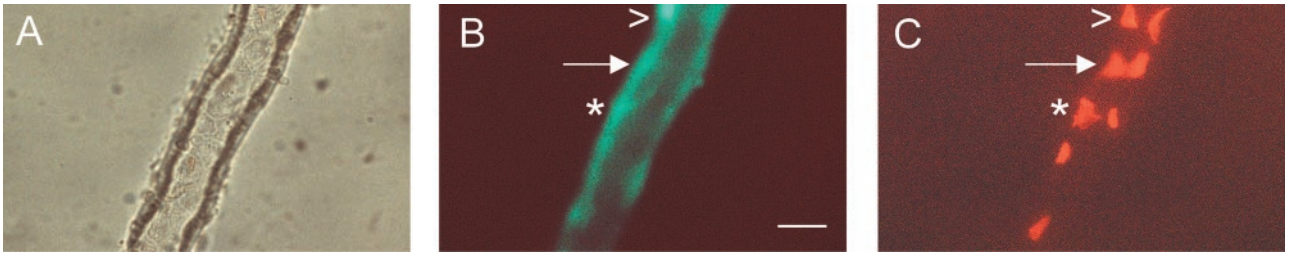

Fig. 1. Light (*A*) and fluorescence (*B*, *C*) micrographs of a single cortical collecting duct (CCD) showing accumulation of fura 2 (*B*) and selective apical binding of rhodamine-conjugated peanut lectin (PNA; *C*) to a unique population of cells. In the fluorescence micrographs, identical symbols identify the doubly stained cells, i.e., PNA-binding intercalated cells. Bar = 30 μm.

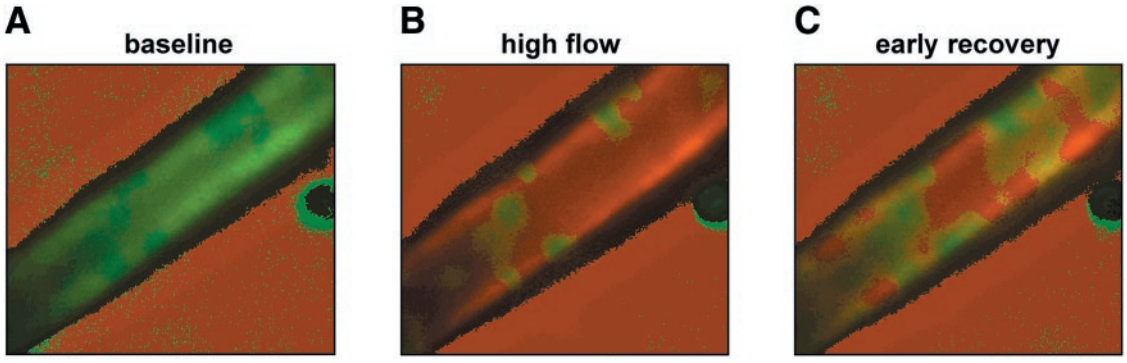

Fig. 2. Pseudocolor fluorescence images of a fura 2-loaded microperfused CCD subject to an acute increase in luminal flow rate. *A*: at baseline, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) averaged ~100 nM, estimated from the 340/380-nm fluorescence excitation ratio measured over individually identified cells. *B*: in response to the increase in luminal flow rate, sufficient to increase tubular diameter by ~20%, $[Ca^{2+}]_i$ increased in most, but not all, cells; the red color corresponds to a $[Ca^{2+}]_i$ of ~300 nM in this experiment. *C*: with recovery, $[Ca^{2+}]_i$ rapidly returned to baseline.

all CCDs were perfused at slow (~1–2 nl·min^{-1}·mm^{-1}) flow rates for 30 min before adding luminal nucleotides. Because fluorescence experiments were performed in the dark, we were unable to accurately measure flow rates, as traditionally measured by obtaining timed 10- to 20-nl collections of tubular fluid (55, 66).

Effect of α,β-methylene-ATP, benzoyl-benzoyl-ATP, 2-methylthio-ATP, or UDP on $[Ca^{2+}]_i$. To determine whether P2X, $P2X_7$, $P2Y_1$, or $P2Y_6$ receptors exist on the apical membrane of principal or intercalated cells, the response of individually identified cells to luminal exposure to α,β-methylene-ATP, BzBz-ATP, 2-methylthio-ATP, or UDP was examined. No significant changes in $[Ca^{2+}]_i$ were seen after luminal perfusion with 100 μM α,β-methylene-ATP (n = 3), BzBz-ATP (n = 4), 2-methylthio-ATP (n = 3), or UDP (n = 4) in either cell type. Representative tracings of these experiments are shown in Fig. 4, *C-F*.

Contribution of extracellular and intracellular Ca^{2+} to nucleotide-induced $[Ca^{2+}]_i$ transients. An increase in $[Ca^{2+}]_i$ could be due to Ca^{2+} release from intracellular stores and/or external Ca^{2+} entry. To evaluate whether the nucleotide-induced increase in $[Ca^{2+}]_i$ was due to Ca^{2+} release from internal stores, the effect of luminal UTP on $[Ca^{2+}]_i$ was examined in CCDs pretreated with either thapsigargin, which depletes internal stores, or 2-APB, an IP_3 receptor antagonist. These studies were performed with UTP because luminal perfusion of this nucleotide consistently led to $[Ca^{2+}]_i$ transients in the CCD.

Pretreatment of CCDs (n = 5) with thapsigargin (100 nM) added to the bathing medium for 30 min led to significant ($P < 0.05$) increases in resting $[Ca^{2+}]_i$ from a baseline of 89 ± 7 to 230 ± 43 nM in principal cells and 124 ± 33 to 284 ± 31 nM in intercalated cells within 3–5 min, consistent with release of internal Ca^{2+} stores. Luminal perfusion of these thapsigargin-treated CCDs with UTP failed to induce an increase in $[Ca^{2+}]_i$ in either principal (to peak of 260 ± 51 nM; P = NS compared with pre-UTP values) or intercalated (to peak of 253 ± 32 nM; P = NS compared with pre-UTP values) cells. Figure 7*A* shows a representative tracing of the effect of UTP on $[Ca^{2+}]_i$ in a thapsigargin-treated CCD. These data suggest that release of intracellular Ca^{2+} stores contributes to the purinergic response.

Addition of 10 μM 2-APB to the bathing medium for 30 min (n = 4) did not significantly alter resting $[Ca^{2+}]_i$ in either principal (117 ± 19 to 138 ± 14 nM; P = NS) or intercalated (103 ± 20 to 132 ± 6 nM; P = NS) cells. Luminal perfusion of 2-APB-treated CCDs with UTP caused an insignificant rise in $[Ca^{2+}]_i$ in both principal (to peak of 170 ± 31 nM; P = NS) and intercalated (to peak of 152 ± 32 nM; P = NS) cells. Figure 7*B* shows a representative tracing of the effect of UTP on $[Ca^{2+}]_i$ in a 2-APB-treated CCD. These data suggest that IP_3 receptor activation participates in the purinergic response.

To evaluate whether the nucleotide-induced increase in $[Ca^{2+}]_i$ requires external Ca^{2+} entry across the apical membrane, the effect of luminal UTP on $[Ca^{2+}]_i$ was examined in CCDs perfused with a nominally Ca^{2+}-free perfusate (Fig. 7*C*) or pretreated for 3–5 min with luminal nifedipine (20 μM), an inhibitor of L-type

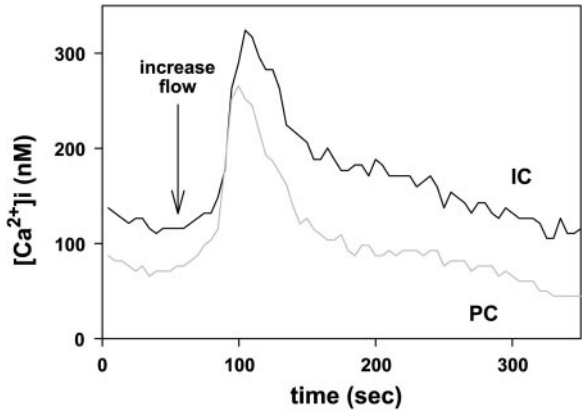

Fig. 3. Representative tracings of the effect of an acute increase in luminal flow rate on $[Ca^{2+}]_i$ in a principal (thin line) and intercalated (thick line) cell in a single CCD. $[Ca^{2+}]_i$ rapidly increased in both cells with the flow-induced increase in tubular diameter, returning to baseline within ~1 min.

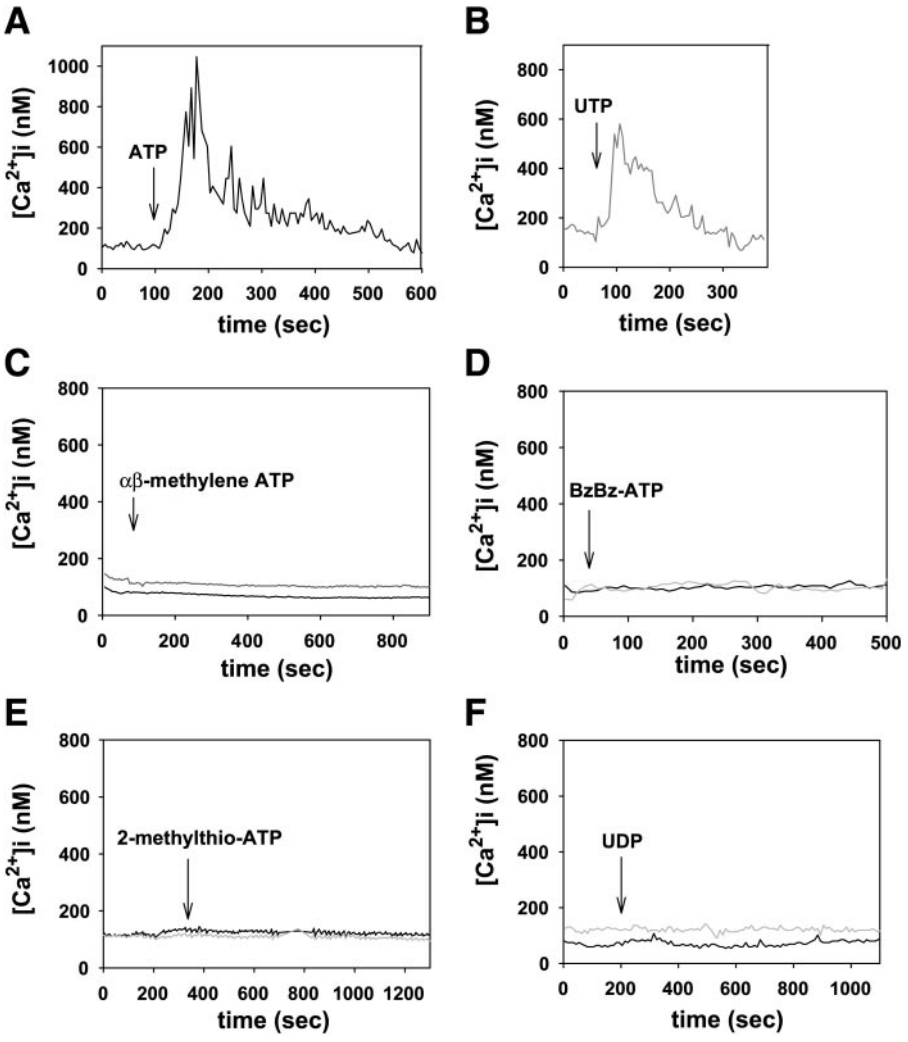

Fig. 4. Representative tracings of luminal nucleotide-induced changes in $[Ca^{2+}]_i$ in principal (thin line) and intercalated (thick line) cells in microperfused CCDs. Luminal ATP (*A*) and UTP (*B*) induced $[Ca^{2+}]_i$ transients in both CCD cell types, consistent with nucleotide activation of apical P2 receptors. Neither α,β-methylene-ATP (*C*), benzoyl-benzoyl-ATP (*D*), 2-methylthio-ATP (*E*), or UDP (*F*), which are selective agonists of the P2X, $P2X_7$, $P2Y_1$, and $P2Y_6$ receptors, respectively, had an effect on $[Ca^{2+}]_i$ in either cell type.

Ca^{2+} channels (Fig. 7*D*). Perfusion of CCDs ($n = 3$) with a Ca^{2+}-free perfusate for 30 min blocked the $[Ca^{2+}]_i$ response to luminal UTP in both cell types. These results are similar to those reported by Nilius et al. (37) who observed that the ATP-induced increase in $[Ca^{2+}]_i$ was inhibited in A6 cells preincubated for >30 min in Ca^{2+}-free bath solutions; a shorter exposure to Ca^{2+}-free medium, however, led to a reduction in the plateau phase but not the initial, rapid rise in $[Ca^{2+}]_i$ (4, 30, 36, 37). Luminal perfusion with nifedipine ($n = 5$) had no effect on resting $[Ca^{2+}]_i$ in either principal (67 ± 8 to 83 ± 15 nM; P = NS) or intercalated (74 ± 10 to 102 ± 18 nM; P = NS) cells. Addition of luminal UTP to these CCDs caused an approximately twofold increase in peak $[Ca^{2+}]_i$ (to 176 ± 27 nM in principal and 200 ± 27 nM in intercalated cells; $P < 0.05$ compared with baseline). This response suggests that the nucleotide-induced increase in $[Ca^{2+}]_i$ requires extracellular Ca^{2+} entry across the luminal membrane.

Effect of ATP/UTP on gap junctional intercellular communication. The similar $[Ca^{2+}]_i$ transients observed in principal and intercalated cells in response to luminal nucleotides suggests that either purinergic receptors are present on both cell types or receptor signaling leads to intercellular spread of Ca^{2+} (or other signaling molecules) between individual collecting duct cells. To determine whether principal and intercalated cells are directly coupled and/or gap junctions are opened in response to nucleotide binding, individual CCD cells in split-open CCDs were microinjected with the cell-impermeant fluorescent dye Lucifer yellow. Under control conditions (Figs. 8, *A* and *B*), Lucifer yellow fluorescence was restricted to the cells in which the tracer was microinjected. Addition of UTP had no apparent effect on intercellular spread in cells monitored for up to 30 min (Fig. 8*C*; $n = 2$ CCDs).

DISCUSSION

Our observations that luminal perfusion of CCDs with ATP, UTP, or ATP-γ-S leads to a suramin-sensitive transient increase in $[Ca^{2+}]_i$ in both principal and intercalated cells in the mammalian CCD (Figs. 4–6) suggests that functional $P2Y_2$ receptors are localized to the apical membranes of both cell types. This is in accordance with the detection of functional $P2Y_2$ receptors on the apical membrane of MDCK cells (17, 69), A6 distal cells (2, 36, 37), primary cultures of rabbit CCD

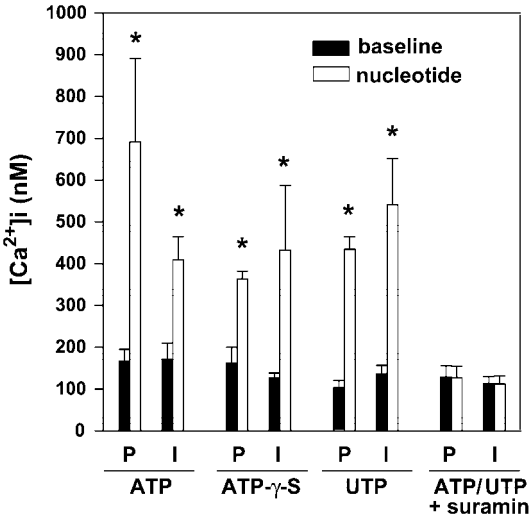

Fig. 5. Mean baseline (filled bars) and peak (open bars) nucleotide-induced $[Ca^{2+}]_i$ detected in principal (P) and intercalated (I) cells in control or suramin-pretreated CCDs. At baseline, $[Ca^{2+}]_i$ did not differ between principal and intercalated cells. Both cell types responded to luminal perfusion of ATP (in 5 of 10 CCDs), ATP-γ-S (in 3/3 tubules), or UTP (in 8/8 tubules) with significant increases in $[Ca^{2+}]_i$ (*$P < 0.05$ compared with baseline). The nucleotide-induced increases in $[Ca^{2+}]_i$ were completely blocked by pretreatment of CCDs with luminal suramin. Values are means ± SE.

cells (30), and immortalized rabbit distal convoluted tubular cells (4, 50). Although $P2Y_4$ receptors also have a high affinity for ATP and UTP, the lack of response to UDP and sensitivity of the $[Ca^{2+}]_i$ response to suramin suggests that functional $P2Y_4$ receptors are not present along the apical membrane of the CCD (5, 64). The absence of an effect of luminal α,β-methylene-ATP (Fig. 4*C*), BzBz-ATP (Fig. 4*D*), 2-methylthio-ATP (Fig. 4*E*), and UDP (Fig. 4*F*) on $[Ca^{2+}]_i$ further suggests that functional P2X, $P2X_7$, $P2Y_1$, and $P2Y_6$ receptors, respectively, are absent on the apical membrane of both cell types.

In contrast to our findings, Deetjen et al. (12) failed to demonstrate apical ATP/UTP-induced effects on $[Ca^{2+}]_i$ in isolated perfused rabbit CCDs. They did, however, detect functional $P2Y_2$ receptors in the mouse CCD in the same study. We consider the discrepant results to reflect species and/or methodological differences between the microperfusion assays used by us and those reported by Deetjen. Specifically, the present studies were performed in CCDs isolated from New Zealand White rabbits and equilibrated in 95% O_2-5% CO_2 at 37°C for 60 min, a treatment period that our laboratory (55, 66) and others (7) have shown necessary for isolated microperfused segments to attain a stable transepithelial voltage and rate of transport. In addition, we found that the response to luminal nucleotides was dependent on flow rate. Specifically, neither principal or intercalated cells responded to luminal nucleotides with an increase in $[Ca^{2+}]_i$ when the CCDs were initially perfused at fast flow rates. Whether the rabbit tubules studied by Deetjen et al. (12) were perfused at comparable flow rates to those used in the present study is uncertain.

The concomitant nucleotide-induced rise in $[Ca^{2+}]_i$ in both principal and intercalated cells can be interpreted to reflect either the presence of apical receptors on both cell types or a nucleotide-induced increase in cell-cell coupling. Whereas principal and intercalated cells have been considered not to be functionally coupled (31, 54), recent studies report the presence of transcripts encoding the gap junctional protein connexin-42 and immunodetectable protein in the CCD (3, 25). These results suggest that, under certain conditions, cell-cell coupling may be activated. The absence of apparent dye coupling between cells in split-open CCDs (Fig. 9) suggests that coupling is absent under baseline conditions. Nor do gap junctions appear to be opened in response to nucleotide exposure.

Of particular note and interest was our incidental finding that an acute increase in tubular fluid flow rate (Figs. 2 and 3) led to a transient increase in $[Ca^{2+}]_i$, a response apparently not mediated by apical P2 purinergic receptor signaling. Praetorius and Spring (42) recently reported that the primary apical cilium in Madin-Darby canine kidney (MDCK) cells is mechanically sensitive, responding to flow with an increase in $[Ca^{2+}]_i$. The Ca^{2+} signal then spreads to adjacent MDCK cells by diffusion of a second messenger through gap junctions (42). Whereas mechanical perturbation of the apical cilium (15) in the microperfused CCD could account for our detection of a flow-stimulated increase in principal cell $[Ca^{2+}]_i$, the mechanism underlying the flow-induced $[Ca^{2+}]_i$ transient in intercalated cells, which are devoid of an apical cilium and appear not to be coupled to adjacent principal cells, remains to be explained.

Mechanical stress results in release of ATP and UTP in polarized airway epithelia across both apical and basolateral membranes (26). Nucleotide release across the apical membrane is proposed to coordinate airway mucociliary clearance responses, including water secretion and ciliary beat frequency, whereas basolateral

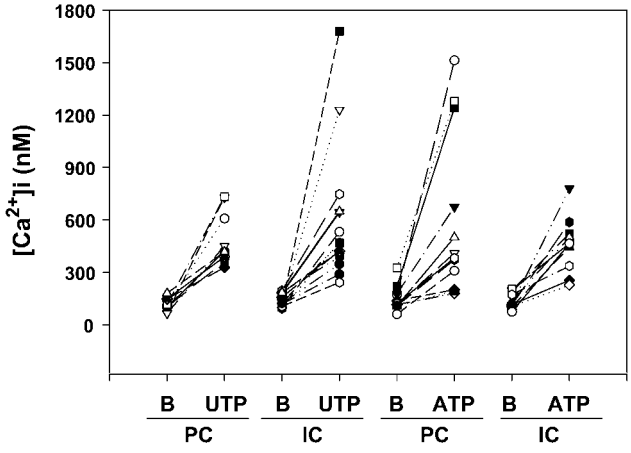

Fig. 6. Scatterplot of changes in $[Ca^{2+}]_i$ in all individually identified principal (PC) and intercalated (IC) cells in the same CCDs as in Fig. 5, studied before (B) and after luminal perfusion with UTP or ATP/ATP-γ-S. Data from the 5 (of 10) CCDs that did not respond to luminal ATP with an increase in $[Ca^{2+}]_i$ (see Fig. 5 legend) are not included.

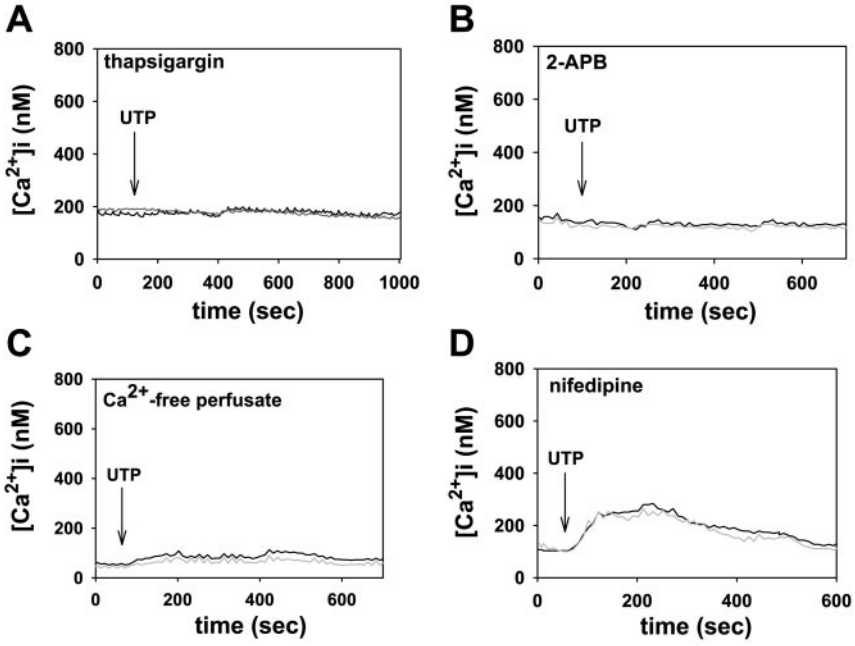

Fig. 7. Representative tracings of luminal nucleotide-induced changes in $[Ca^{2+}]_i$ in principal (thin line) and intercalated (thick line) cells in CCDs pretreated with inhibitors of internal Ca^{2+} mobilization or extracellular Ca^{2+} entry. Depletion of internal Ca^{2+} stores, either by pretreatment with thapsigargin (*A*) or the IP_3 receptor antagonist 2-APB (*B*), blocked the expected response to purinergic receptor activation. Luminal perfusion with a nominally Ca^{2+}-free perfusate (*C*) or the L-type Ca^{2+} channel blocker nifedipine (*D*) also abrogated the nucleotide-induced increase in $[Ca^{2+}]_i$.

release represents a paracrine mechanism by which mechanical stresses signal adjacent epithelial cells (26). To the extent that mechanical stress induced by tubular fluid flow results in bidirectional nucleotide release, the absence of effect of luminal suramin on the flow-induced $[Ca^{2+}]_i$ transient is compatible with the possibility that nucleotides released at the basolateral membrane bind to and activate basolateral P2 purinergic receptors. Although luminal activation of apical P2X receptors could also account for the flow-induced response, our data suggest that this class of receptors is nonfunctional, if present, on the apical membrane. Also possible, but not explored in the present study, is that P1 purinergic receptor signaling contributes to the flow-induced responses.

We believe that the response to luminal perfusion with nucleotides reflects activation of luminal $P2Y_2$ receptors and is distinct from the response to increasing flow/tubule stretch because of the following observations. First, perfusion with suramin inhibited the $[Ca^{2+}]_i$ response to luminal nucleotides but not tubule stretch. Second, repetitive increases in tubular flow rate led to multiple transient increases in $[Ca^{2+}]_i$, whereas tubules were insensitive to more than one sequential challenge with luminal nucleotide (data not shown).

Our data suggests that the apical purinergic receptor-induced $[Ca^{2+}]_i$ transient comprises at least two interdependent components: a rapid mobilization of Ca^{2+} from IP_3-sensitive stores and luminal Ca^{2+} entry through nifedipine-sensitive Ca^{2+} channels. The inhibition of the nucleotide-induced response by thapsigargin (Fig. 7*A*) and 2-APB (Fig. 7*B*) is consistent with stimulation of the IP_3 receptor and internal Ca^{2+} mobilization. The absence of a response in cells subjected to prolonged exposure to Ca^{2+}-free luminal perfusate (Fig. 7*C*), which presumably depletes internal stores, provides additional evidence for the participation of this pathway in apical purinergic signaling. Whether the nifedipine-sensitive extracellular Ca^{2+} influx step is mediated by a Ca^{2+}-release-activated Ca^{2+} channel, an L-type Ca^{2+} channel or Ca^{2+}-permeable cation

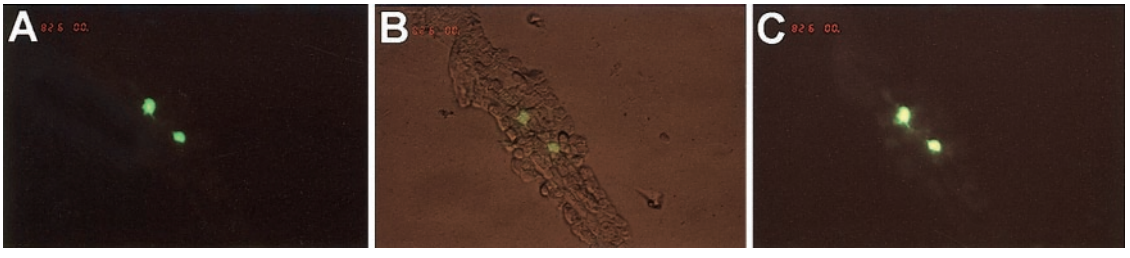

Fig. 8. Effect of UTP on cell-cell coupling in the CCD. Two cells in a split-open CCD (apical surface exposed) were microinjected with Lucifer yellow, as described in MATERIALS AND METHODS. When examined by epifluorescence illumination (in *A* at 490-nm excitation; with coincident light in *B*) 5 min later, there was no evidence of intercellular spread or significant leakage of the dye. Thus CCD cells appear not be directly coupled at baseline. There was no detectable spread of Lucifer yellow into adjacent cells for up to 30 min (15-min image shown in *C*) after addition of UTP to the bath (to final concentration of ~100 μM), consistent with the absence of nucleotide-induced coupling.

channels (8, 16) remains to be established. The inhibition of this Ca^{2+} entry step by 2-APB alone (Fig. 7*B*) is compatible with nucleotide-induced Ca^{2+} entry via store-operated channels (SOCs), activated by a reduction in Ca^{2+} in the endoplasmic reticulum lumen (35, 38, 45) and suggests that the IP_3 receptor is essential for maintaining coupling between store depletion and activation of SOCs. Ma et al. (35) recently suggested that store emptying promotes a reversible docking of the endoplasmic reticulum with the plasma membrane to activate SOCs (40, 44).

The physiological relevance of apical P2 receptors in the CCD depends on delivery of the appropriate ligand to the extracellular space and the local activity of ecto-nucleotidases, which catalyze the catabolism of ATP. Collecting duct cells may constitutively release ATP into the extracellular space in concentrations sufficient to activate P2 receptors (13, 23, 27, 33, 39, 43, 47, 48, 57, 61, 63, 65). The exclusive localization of ecto-5′-nucleotidase at the luminal membrane of intercalated but not principal cells (21) suggests that ATP, if released locally into the lumen of the CCD, may participate in P1 receptor signal transduction.

An increasing number of functional studies demonstrate that activation of apical purinergic receptors in the kidney leads to significant alterations in ion and solute transport. Relevant to the CCD are the observations that apical (or basolateral) ATP inhibits electrogenic amiloride-sensitive Na^+ absorption in rabbit (30) and M-1 mouse (11) CCD cell lines. Apical (or basolateral) ATP stimulates Cl^- secretion in M-1 mouse CCD cells (11) and MDCK cells (67). Furthermore, it has been shown that nucleotides activate the inwardly rectifying ~70-pS K channel in MDCK cells (19, 20, 41) and ~32-pS K channel in A6 cells (37), but inhibit the apical small conductance K channels in mouse CCD (34). Finally, basolateral nucleotides inhibit vasopressin-induced water transport in perfused rabbit CCDs (49). Whereas our identification of functional P2Y receptors on principal cells is compatible with their proposed role in regulating Na^+, K^+, and Cl^- transport, the detection of these purinergic receptors on the apical surfaces of intercalated cells raises the possibility that H^+/HCO_3^- transport by this population of cells may also be modulated by luminal nucleotides.

In conclusion, functional $P2Y_2$ receptors are present on the apical surfaces of both principal and intercalated cells in the microperfused rabbit CCD. The $[Ca^{2+}]_i$ transients observed in these cells in response to luminal nucleotides is apparently not due to intercellular coupling. In addition, the $[Ca^{2+}]_i$ transients induced by acute increases in luminal flow rate/epithelial stretch is not mediated by apical suramin-sensitive purinergic activation. The localization of functional P2 receptors to the apical membrane of both principal and intercalated cells suggests that luminal ATP and/or UTP may play a significant regulatory role in electrolyte, acid-base, and water transport in the CCD.

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