J. Membrane Biol. 183, 115–124 (2001)
DOI: 10.1007/s00232-001-0059-4

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 2001

$P2Y_2$ Receptor-mediated Inhibition of Amiloride-sensitive Short Circuit Current in M-1 Mouse Cortical Collecting Duct Cells

J. Thomas[1], P. Deetjen[1], W.H. Ko[2], C. Jacobi[1], J. Leipziger[1]
[1]Physiologisches Institut, Albert-Ludwigs-Universität, Hermann-Herder-Straße 7, 79104 Freiburg, Germany
[2]Department of Physiology, The Chinese University of Hong Kong, New Territories, Hong Kong

Received: 9 February 2001/Revised: 17 May 2001

Abstract. Extracellular nucleotides modulate renal ion transport. Our previous results in M-1 cortical collecting duct cells indicate that luminal and basolateral ATP via $P2Y_2$ receptors stimulate luminal Ca^{2+}-activated Cl^- channels and inhibit Na^+ transport. Here we address the mechanism of ATP-mediated inhibition of Na^+ transport. M-1 cells had a transepithelial voltage (V_{te}) of −31.4 ± 1.3 mV and a transepithelial resistance (R_{te}) of 1151 ± 28 Ωcm^2. The amiloride-sensitive short circuit current (I_{sc}) was −28.0 ± 1.1 $\mu A/cm^2$. The ATP-mediated activation of Cl^- channels was inhibited when cytosolic Ca^{2+} increases were blocked with cyclopiazonic acid (CPA). Without CPA the ATP-induced $[Ca^{2+}]_i$ increase was paralleled by a rapid and transient R_{te} decrease (297 ± 51 Ωcm^2). In the presence of CPA, basolateral ATP led to an R_{te} increase by 144 ± 17 Ωcm^2 and decreased V_{te} from −31 ± 2.6 to −26.6 ± 2.5 mV. I_{sc} dropped from −28.6 ± 2.4 to −21.6 ± 1.9 $\mu A/cm^2$. Similar effects were observed with luminal ATP. In the presence of amiloride, ATP was without effect. This reflects ATP-mediated inhibition of Na^+ absorption. Lowering $[Ca^{2+}]_i$ by removal of extracellular Ca^{2+} did not alter the ATP effect. PKC inhibition or activation were without effect. Na^+ absorption was activated by pH_i alkalinization and inhibited by pH_i acidification. ATP slightly acidified M-1 cells by 0.05 ± 0.005 pH units, quantitatively not explaining the ATP-induced effect. In summary this indicates that extracellular ATP via luminal and basolateral $P2Y_2$ receptors inhibits Na^+ absorption. This effect is not mediated via $[Ca^{2+}]_i$, does not involve PKC and is to a small part mediated via intracellular acidification.

Correspondence to: J. Leipziger
leipziger@fi.au.dk

Key words: M-1 cell — Cortical collecting duct — ENaC — ATP — P2-Receptor — Na^+-Transport

Introduction

Epithelial cells frequently express a variety of P2-receptors in the luminal and basolateral membrane [14, 15, 20, 21]. Like other membrane receptor families, P2-receptors are subdivided into metabotropic (G-protein-coupled) P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$) and ionotropic (nonselective Ca^{2+}-permeable cation channel) P2X ($P2X_{1-7}$) receptors [33]. P2 receptors are activated by extracellular nucleotides, most commonly ATP, UTP, ADP or UDP. In the mammalian renal tubular system, evidence for the expression of P2-receptors along the entire nephron has been presented [1, 4, 24, 26]. Our understanding of ATP's functional significance in the nephron or other epithelia, however, remains largely obscure. Renal tubular cells most commonly express P2Y receptors, predominantly coupled to the $InsP_3$/Ca^{2+} signaling cascade [1, 4, 6]. Evidence has also been presented compatible with the expression of P2X receptors e.g., in mIMCD-K2 cells [26]. In rat inner medullary collecting duct the expression of luminal and basolateral $P2Y_2$ receptors has been proposed [17] and the same group and others demonstrated in intact tubules that basolateral ATP inhibits ADH-stimulated H_2O transport [16, 35]. In a previous paper we used mouse M-1 cortical collecting duct cells to investigate the effect of luminal and basolateral ATP and other nucleotides on ion transport in Ussing chamber experiments [2]. M-1 cells have previously been shown to be a suitable cell line with close functional similarity to cortical collecting duct principal cells [37]. As monolayers on filter membranes they express epithelial sodium channels (ENaC)

and exhibit an amiloride-sensitive short circuit current in the range of 20–50 $\mu A/cm^2$ [2, 37]. Previous experiments showed that ATP or UTP via a luminal and basolateral $P2Y_2$ receptor and a subsequent $[Ca^{2+}]_i$ increase activate a Cl^- conductance in the luminal membrane [2]. Our data indicated that extracellular nucleotides in addition inhibit the amiloride-sensitive short circuit current. A previous study from rabbit connecting tubule cells similarly identified luminal and basolateral $P2Y_2$ receptors. Here the nucleotides ATP and UTP inhibited Na^+ and Ca^{2+} absorption [18]. The authors suggest that inhibition of amiloride-sensitive short circuit current was independent of an increase in $[Ca^{2+}]_i$. Other data, however, in isolated rabbit CCD tubules, indicate that Na^+ absorption is inhibited in a $[Ca^{2+}]_i$-dependent manner [8, 13, 38].

In this study we specifically investigate the $P2Y_2$-mediated effect on the amiloride-sensitive short circuit current and its mechanism of inhibition in mouse M-1 cortical collecting duct principal cells. For this purpose we simultaneously measured the amiloride-sensitve short circuit current and the intracellular Ca^{2+} activity in a specially designed Ussing chamber.

Materials and Methods

Cell Culture

M-1 CCD cells (kindly provided by Dr. C. Korbmacher, Oxford, Great Britain) have been developed from a mouse transgenic for the early region of simian virus 40, Tg(SV40E)Bri/7 [37]. M-1 cells show many characteristics of the cortical collecting duct when grown in monolayers with a high transepithelial resistance (R_{te}) and a lumen negative transepithelial voltage (V_{te}). As described below, our measured R_{te} and V_{te} values were consistent with the original publication [37]. M-1 cells were grown on collagen/fibronectin-coated plastic flasks in DMEM-Ham's F-12 medium (Life Technologies, Karlsruhe, Germany), to which 100 ml/l fetal calf serum (FCS), 100 mg/l penicillin/streptomycin and 0.5 µmol/l dexamethasone was added. Inverted filter membranes (Transwell®-Col filter, 12 mm diameter, 0.4 µm pore size, Costar, Bodenheim, Germany) were immersed in culture medium into a 4 cm deep special cup. Subsequently cells were seeded onto the lower side of the filter membrane. This allows an unobstructed optical access and visualization of single cellular fluorescence in the inverted microscope. After 2 days the filters were turned around and placed in 24-well culture plates (Costar, Bodenheim, Germany). During culture the luminal and the basolateral side were covered with medium.

Ussing Chamber Experiments

To measure the electrogenic Na^+ transport, the Ussing chamber technique was applied. This technique allows for the measurement of transepithelial voltage (V_{te}) and transepithelial resistance (R_{te}). Our chamber had an aperture of 1 cm^2. The measurements were performed in 'open-circuit' mode. V_{te} was referred to the basolateral side. R_{te} was calculated from the voltage deflections induced by short (0.6 sec) current pulses [23]. These deflections were corrected for those obtained with the empty chamber with a filter membrane. The equivalent short circuit current was obtained by Ohm's law ($I_{sc} = V_{te}/R_{te}$). The calculated I_{sc} changes were derived from peak values. An important feature of this specially designed chamber is that it permits the use of fluorescent indicator dyes to measure, e.g., $[Ca^{2+}]_i$ in parallel with I_{sc}.

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

Measurement of $[Ca^{2+}]_i$ was performed with the Ca^{2+}-dye fura-2-AM using single-photon counting as described previously [29]. The studies were performed on an inverted microscope (Axiovert 10, Zeiss, Jena, Germany). Before mounting the cells into the experimental chamber, confluence was evaluated by transmission optics and by measuring R_{te} and V_{te} in the filter cup with the Epithelial Voltohmmeter (EVOM® Stix-electrode, World Precision Instruments, USA). The above-mentioned chamber was used to measure simultaneously $[Ca^{2+}]_i$, R_{te} and V_{te}. A filter was positioned in this chamber with the cells (luminal side) facing downside. The cells grown on Transwell® Col filters were used at day 3–5. Autofluorescence of the cells and Transwell® Col filters amounted to app. 10% of the entire fluorescence signal at the beginning of the experiment and was subtracted. A long-distance objective (LD-Achroplan 40×/0.6, Zeiss, Germany) was used to visualize the cells. The distance from the glass base to the cell layer was 2 mm. In this chamber the upper "basolateral" side was freely accessible. The cells were loaded for 60 min in a 5% CO_2/95% O_2 atmosphere at 37°C in the modified culture medium to which fura-2-AM (10 µM), pluronic F127 (1.6 µM) and probenecid (5 mM) had been added. Pluronic F127 is a surfactant polyol that helps to solubilize water-insoluble dyes like fura-2-AM. Probenecid has been added to the incubation-solution to achieve an improved dye-loading of the cells. As a measure of $[Ca^{2+}]_i$ the fluorescence emission ratio at 340nm/380nm excitation was calculated. In each experiment the fluorescence signal was recorded from approximately 10 cells. The closed side of the Ussing chamber had a "luminal" chamber volume of 200 µl and the open "basoleteral" side, some 500 µl. Both sides were continuously perfused with a flow rate of approximately 2 ml/min. All experiments were performed at 37°C. The fura-2 $[Ca^{2+}]_i$ data will be shown qualitatively as a change in fluorescence ratio.

Measurement of pH_i

The intracellular pH was measured with the pH-dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) using the same experimental setup as described above. The cells were loaded for 30 min in a 5% CO_2/95% O_2 atmosphere at 37°C in culture medium to which BCECF-AM (1 µM), pluronic F127 (1.6 µM) and probenecid (5 mM) had been added. As a measure of pH_i the fluorescence emission ratio at 488nm/436nm excitation was calculated. Calibration of the fluorescence signal was performed with solutions of different pH containing high K^+ (145 mM) and the K^+/H^+ exchanger nigericin (4 µM). Under these conditions the intracellular pH (pH_i) is clamped to that of the extracellular solution. Calibration curves were linear within a pH_i range from 6.5 to 7.8.

Solutions and Chemicals

Pluronic F127, fura-2-AM and BCECF-AM were obtained from Molecular Probes (Eugene, Oregon, USA). All other chemicals were of the highest grade of purity available and were obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). The experiments were performed with the following solution (in mM): 145 NaCl, 1 $MgCl_2$, 1.3 Ca-gluconate, 5 D-glucose, 0.4 KH_2PO_4, 1.6 K_2HPO_4. The 10 µM Ca^{2+}-solution contained (in mM) 145 NaCl, 1

$MgCl_2$, 0.1 Ca-gluconate, 5 D-glucose, 0.4 KH_2PO_4, 1.6 K_2HPO_4. To all solutions 5 mM probenecid was added. All solutions were titrated to pH 7.4.

Statistics

The data shown are either original traces or mean values ± SEM (*n*), where *n* refers to the number of experiments. A paired *t*-test was used to compare mean values within one experimental series. A *p* value of <0.05 was accepted to indicate statistical significance.

Abbreviations

PMA, Phorbol 12-myristate 13-acetate; ENaC, epithelial Na^+ channel; CPA, cyclopiazonic acid; CCD, cortical collecting duct; BIM, Bisindolylmaleimide.

Results

Similar to previous studies, confluent M-1 cells had properties of a very tight epithelium. In the presence of 0.1 μM dexamethasone the mean resting R_{te} was 1151 ± 28 Ωcm^2. V_{te} amounted to −31.4 ± 1.3 mV (lumen negative) and the resulting calculated short circuit current (I_{sc}) was −28.0 ± 1.1 $\mu A/cm^2$ ($n = 95$). The I_{sc} was almost completely and reversibly inhibited by luminal amiloride (10 μM) (−3.05 ± 0.61 $\mu A/cm^2$). Luminal amiloride reversibly increased R_{te} from 970 ± 59 to 1170 ± 81 Ωcm^2 ($n = 8$). Basolateral amiloride (100 μmol/l) had no effect on I_{sc} ($n = 5$).

$[Ca^{2+}]_i$ Store Depletion Unmasks the ATP-induced Inhibition of Amiloride-sensitive Short Circuit Current

Figure 1*A* shows an original Ussing chamber experiment with the simultaneous measurement of $[Ca^{2+}]_i$. From top to bottom the recordings of the transepithelial voltage (V_{te}), the transepithelial resistance (R_{te}), the corresponding short circuit current (I_{sc}) and the fura-2 fluorescence ratio 340nm/380nm are depicted. The application of basolateral ATP (100 μM) induced a rapid $[Ca^{2+}]_i$ rise with an initial peak and a secondary plateau (mean fluorescence 340nm/380nm peak ratio increase: 0.45 ± 0.07, $n = 6$). Similar to the data presented in the previous study [2], basolateral ATP reversibly reduced V_{te} from −35.1 ± 5.2 mV to −20.2 ± 4 mV and reduced R_{te} from 1275 ± 46 to 805 ± 42 Ωcm^2. I_{sc} initially showed a drop from −27.3 ± 3.5 to −24 ± 3.6 $\mu A/cm^2$, parallel to the decrease in R_{te} ($n = 6$). Similar results were obtained with the addition of luminal ATP (*data not shown*). The previous work showed that ATP and UTP were equipotent. Furthermore, the expression of $P2Y_2$-receptor mRNA was demonstrated. Therefore, the ATP effect is most likely mediated via this P2Y-receptor subtype located in the luminal and basolateral membrane of M-1 CCD cells [2]. In the preceding paper [2] a number of arguments were presented indicating that ATP and UTP stimulate Ca^{2+}-activated Cl^- channels in the luminal membrane. Furthermore, inhibition of amiloride-sensitive I_{sc} by ATP was suggested. Here we investigate this phenomenon in detail. To investigate this we tried to inhibit the Ca^{2+}-activated Cl^- conductance by depletion of intracellular $[Ca^{2+}]_i$ stores. This was successfully achieved with, e.g., basolateral cyclopiazonic acid (CPA, 5 μM), a well described inhibitor of sarcoplasmatic/endoplasmatic reticulum Ca^{2+} ATPases (SERCA) [27]. This is a widely used protocol to prevent $InsP_3$-mediated $[Ca^{2+}]_i$ elevations in nonexcitable cells. As seen in Fig. 1*A,* addition of 5 μM basolateral CPA induced a slow increase of $[Ca^{2+}]_i$ (mean fluorescence 340nm/380nm peak ratio increase: 0.34 ± 0.05, $n = 13$). Interestingly, this never altered transepithelial electrical parameters (R_{te}, V_{te}). The subsequent addition of basolateral ATP (100 μM) demonstrated that the intracellular Ca^{2+} stores had been almost completely emptied. Under these conditions no significant drop of R_{te} was measured. In contrast, ATP now increased R_{te} by 144 ± 17 Ωcm^2 ($n = 20$). Inspection of the I_{sc} trace now clearly shows an ATP-induced reversible decrease of I_{sc} (I_{sc} decrease: 7.02 ± 0.59 $\mu A/cm^2$, $n = 20$). A repeated application of basolateral ATP elicited the same response. At the end of the experiment luminal amiloride (10 μM) inhibited Na^+ absorption, reflected in complete inhibition of I_{sc}. Now basolateral ATP (100 μmol/l) elicited no further effect on the electrical transepithelial parameters V_{te} and R_{te} (I_{sc} decrease: 0.37 ± 0.07 $\mu A/cm^2$, $n = 7$). Also basolateral UTP (100 μM) under continuous $[Ca^{2+}]_i$-store depletion inhibited the amiloride-sensitive I_{sc} by 5.01 ± 1.76 $\mu A/cm^2$ ($n = 4$). These data indicate that $[Ca^{2+}]_i$-store depletion inhibits the previously described activation of a luminal Cl^- conductance. Under these conditions the observed effect is consistent with an ATP-mediated inhibition of amiloride-sensitive I_{sc}. The ATP-mediated inhibition of Na^+ transport was always transient as depicted in Fig. 1*B*. In a series of 6 experiments, where basolateral ATP was given for a longer period, a maximal decrease of I_{sc} by −6.92 ± 1.19 $\mu A/cm^2$ was observed after 101.5 ± 4.2 sec. After 4 min the ATP-mediated I_{sc} decrease amounted only to 4.0 ± 1.19 $\mu A/cm^2$.

Luminal ATP Inhibits the Amiloride-sensitive I_{sc}

Subsequently we investigated the effect of luminal ATP on the amiloride-sensitive I_{sc} in M-1 cells. In this and all subsequent experiments the intracellular Ca^{2+} stores were depleted with CPA (5 μM). An original experiment

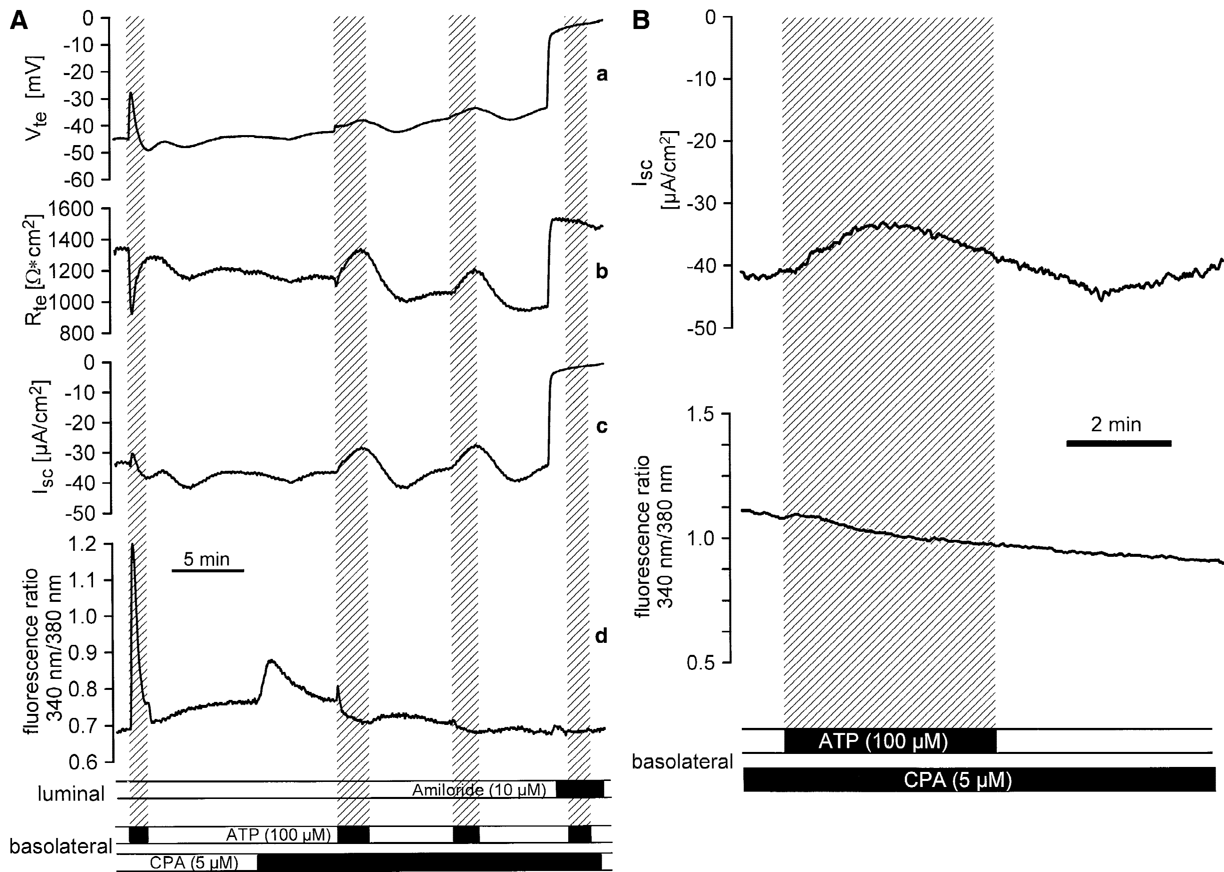

Fig. 1. Effect of basolateral ATP (100 μM) in M-1 cells grown on permeable filters. (*A*) Effect on transepithelial voltage (V_{te}; *a*), transepithelial resistance (R_{te}; *b*), equivalent short circuit current (I_{sc}; *c*) and Fura-2 $[Ca^{2+}]_i$-fluorescence ratio 340nm/380nm (original recording; *d*). Basolateral ATP induced a drop in lumen-negative V_{te}, strongly decreased R_{te}, induced a "complex" I_{sc} response and increased $[Ca^{2+}]_i$. Subsequently, under intracellular Ca^{2+}-store depletion with cyclopiazonic acid (5 μM), the ATP $[Ca^{2+}]_i$ response was inhibited. Now ATP increased R_{te} and induced an inhibition of I_{sc}. This reflects a reduction in Na^+ absorption as seen by the subsequent addition of luminal amiloride (10 μM). Amiloride induced 1) a reduction of lumen-negative V_{te}, 2) an increase in R_{te} and 3) a drop in negative I_{sc}, consistent with near-to-complete inhibition of electrogenic Na^+ absorption. (*B*) *Upper panel:* equivalent short circuit current (I_{sc}). *Lower panel:* Fura-2 $[Ca^{2+}]_i$ fluorescence ratio 340nm/380nm (original recording). Basolateral ATP applied for longer periods (here 4 min) always transiently reduced Na^+ transport.

is shown in Fig. 2. The upper panel shows the I_{sc} and the lower panel the measurement of $[Ca^{2+}]_i$. As obvious, also luminal ATP inhibited the amiloride-sensitive I_{sc}. This experiment shows that the inhibition of amiloride-sensitive I_{sc} by maximal luminal ATP stimulation (100 μM) was smaller (I_{sc} decrease: 2.47 ± 0.45 μA/cm², n = 8) compared to maximal basolateral ATP stimulation (100 μM) (I_{sc} decrease: 7.02 ± 0.59 μA/cm², n = 20). Similar to basolateral ATP also luminal ATP increased R_{te} and decreased V_{te} (*data not shown*). For all further experiments we used the stimulation with basolateral ATP under continuous intracellular Ca^{2+} store depletion with CPA.

Subsequently we investigated the effect of luminal ATP (100 μM) in the presence of 100 μM basolateral ATP. Initially basolateral ATP was added and in this series inhibited I_{sc} by 4.9 ± 1.31 μA/cm². After two minutes of basolateral stimulation luminal ATP induced a further decrease of Na^+ absorption by 2.7 ± 0.54 μA/cm² (n = 5). These results indicate that luminal and basolateral ATP inhibit Na^+ transport additively.

Next we determined the concentration response relationship for basolateral ATP on amiloride-sensitive I_{sc}. Figure 3A shows an original experiment, in which basolateral ATP reduced V_{te} and increased R_{te} in a concentration-dependent manner, resulting in a decrease of the amiloride-sensitive I_{sc}. The summary of these experiments is shown in Fig. 3*B*. The I_{sc}-inhibition (●) (IC_{50}: 4.13 μM) and the corresponding decrease of transepithelial conductance (IC_{50}: 6.34 μM) (G_{te}) (▲) are shown as a function of basolateral ATP concentration. Concentrations resulting in 50% inhibition (IC_{50} values) were obtained by fitting the data to the Michaelis-Menten equation.

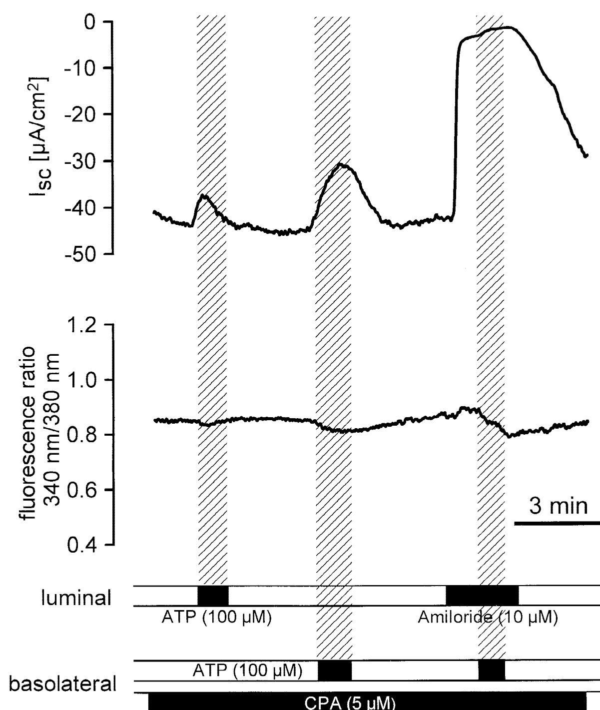

Fig. 2. Effect of basolateral and luminal ATP (100 μM) in M-1 cells grown on permeable filters on equivalent short circuit current (I_{sc}) (*upper panel*) and Fura-2 $[Ca^{2+}]_i$-fluorescence ratio 340nm/380nm (*lower panel,* original recording). Intracellular Ca^{2+} stores were continuously depleted with cyclopiazonic acid (5 μM). As can be seen, the ATP-mediated inhibition of I_{sc} depicted in Fig. 1 was similarly inducible with luminal ATP. Also shown in Fig. 2 is the observation that under luminal amiloride (10 μM) basolateral ATP (100 μM) did not alter I_{sc}.

ATP Inhibits Amiloride-sensitive I_{sc} Independently of $[Ca^{2+}]_i$

The presented data strongly suggest that the ATP-mediated inhibition of Na^+ transport was not mediated via an increase of $[Ca^{2+}]_i$. However, this has been a controversial issue [13, 18]. To investigate this topic more vigorously, we performed the following experiments. Ca^{2+}-store depletion is well known to activate store-operated Ca^{2+}-entry pathways in the basolateral membrane of renal epithelia [11]. Thus, in certain subcellular regions, $[Ca^{2+}]_i$ could be persistently high but undetectable with our fura-2 method. We therefore lowered the extracellular Ca^{2+} concentration to 100 μM on the basolateral side of the M-1 monolayer. This maneuver did not affect transepithelial electrical parameters. As seen in Fig. 4*A*, this produced a substantial and reversible reduction of $[Ca^{2+}]_i$ (mean fluorescence ratio decrease: 0.13 ± 0.02, $n = 5$). In a strictly paired fashion basolateral ATP was now added during the condition of "normal" or "lowered" cytosolic Ca^{2+} and the inhibitory effect on Na^+ transport was assessed. As seen from Fig. 4*A*, lowering $[Ca^{2+}]_i$ did not alter the ATP-mediated inhibition of Na^+ absorption. The data are summarized in Fig. 4*B*. These experiments strongly indicate, that the ATP-induced inhibition of I_{sc} in M-1 cells is regulated independently of $[Ca^{2+}]_i$.

The ATP-induced Inhibition of Amiloride-sensitive I_{sc} is Not Mediated Via PKC

Previous studies in either rat or rabbit distal nephron segments have proposed that distal tubular Na^+ absorption is inhibited via activation of protein kinases C (PKC) [3, 7]. We therefore investigated the involvement of PKC. For this purpose different PKC inhibitors or PKC activators were applied. Figure 5 depicts an original experiment with the PKC inhibitor bisindolylmaleimide (BIM, 1 μmol/l). Ca^{2+}-store depletion was again performed with CPA (5 μM). We compared the effect of basolateral ATP (100 μM) on I_{sc} in the absence and presence of BIM (1 μM). As obvious from Fig. 5, BIM did not affect the ATP-mediated inhibition of amiloride-sensitive I_{sc}. In summary, in this series basolateral ATP inhibited I_{sc} in the pre-control period by 5.03 ± 0.6, with BIM by 3.76 ± 0.48 and after washout of the PKC inhibitor by 3.77 ± 0.45 $\mu A/cm^2$ ($n = 5$). Similarly staurosporine (10^{-7} mol/l), a nonspecific PKC inhibitor, did not affect the ATP-induced decrease of the amiloride-sensitive I_{sc} (*data not shown*). Higher concentrations of staurosporine could not be tested, since this decreased R_{te} dramatically. Subsequently we tested if PKC-activation with PMA (0.1 or 0.5 μM) could mimic the ATP-stimulated inhibition of Na^+ absorption. This was not the case (*data not shown, n* = 5). Furthermore, ATP elicited an undisturbed inhibitory effect on the amiloride-sensitive I_{sc} in the continuous presence of PMA (0.1 or 0.5 μM, *data not shown*). Taken together, these results indicate that PKC is not involved in mediating the ATP-induced inhibition of amiloride-sensitive I_{sc}.

Basolateral and Luminal ATP Acidifies M-1 Cells

It is well known that ENaC is closed by acidic intracellular pH (pH_i) [10]. Since extracellular ATP via P2 receptors can acidify epithelial cells the observed ATP effect could be mediated via an intracellular acidification. Therefore we measured the pH_i in BCECF-loaded M-1 monolayers. Figure 6 shows an original experiment with the simultaneous measurement of R_{te}, I_{sc} and pH_i. Basolateral ATP (100 μM) under CPA caused a small acidification of the pH_i in M-1 cells paralleled by the known inhibition of the amiloride-sensitive I_{sc}. This acidification by basolateral ATP amounted to 0.05 ± 0.005 pH-units ($n = 20$). Basolateral acetate (20 mmol/l) acidified M-1 cells by 0.267 ± 0.048 pH-units and inhibited in parallel the amiloride-sensitive I_{sc} ($n = 11$).

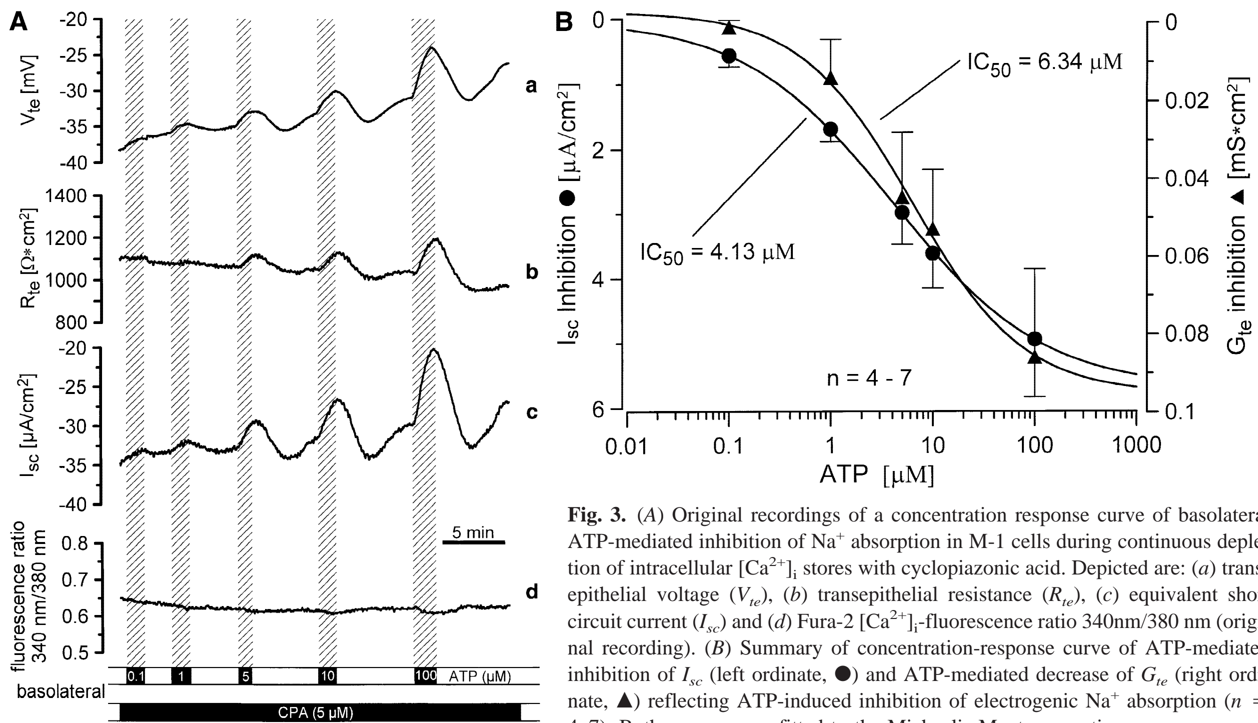

Fig. 3. (*A*) Original recordings of a concentration response curve of basolateral ATP-mediated inhibition of Na^+ absorption in M-1 cells during continuous depletion of intracellular $[Ca^{2+}]_i$ stores with cyclopiazonic acid. Depicted are: (*a*) transepithelial voltage (V_{te}), (*b*) transepithelial resistance (R_{te}), (*c*) equivalent short circuit current (I_{sc}) and (*d*) Fura-2 $[Ca^{2+}]_i$-fluorescence ratio 340nm/380 nm (original recording). (*B*) Summary of concentration-response curve of ATP-mediated inhibition of I_{sc} (left ordinate, ●) and ATP-mediated decrease of G_{te} (right ordinate, ▲) reflecting ATP-induced inhibition of electrogenic Na^+ absorption (n = 4–7). Both curves were fitted to the Michaelis-Menten equation.

Alkalization with luminal NH_4/NH_3 (20 mmol/l) markedly increased I_{sc}. The observation that acidification inhibits and alkalinization activates I_{sc} is highly consistent with previous investigations addressing the issue of pH_i modulation of Na^+ absorption [9, 30]. In our further analysis (Fig. 7) we plotted the change of I_{sc} as a function of pH_i. (▲: a single 'acetate' experiment, ■: a single NH_4/NH_3 experiment and ●: a single ATP experiment). In the range of ± 0.5 pH_i units around resting pH_i a linear correlation was observed. As obvious the ATP-induced effects on I_{sc} and pH_i do not superimpose onto this regression line. We therefore conclude that the ATP-induced acidification does not play a major role as mediator of the inhibition of amiloride-sensitive I_{sc}.

Discussion

Na^+ absorption in the distal mammalian nephron occurs transcellularly through uptake of Na^+ via luminal ENaC channels and subsequent ATP-dependent translocation of Na^+ onto the blood side through the basolateral Na^+/K^+-ATPase. Key activators of this process are aldosterone or ADH. In addition, other hormonal factors modulate electrogenic Na^+ absorption. An activator of Na^+ absorption, e.g., is insulin, which conveys its action via the recently identified serum- and glucocorticoid-induced protein kinase (SGK) [31, 32]. An inhibitor of Na^+ transport is basolateral PGE_2 [12]. The key finding of this paper is that under continuous $[Ca^{2+}]_i$-store depletion luminal and basolateral ATP and UTP inhibited electrogenic Na^+ absorption.

A $P2Y_2$ Receptor-mediated Effect

In our preceding paper we suggested that the nucleotide-mediated effect was via a $P2Y_2$ receptor. The conclusion was based on two arguments: 1) The identification of specific mRNA for this receptor subtype and 2), a typical pharmacology with UTP being as potent as ATP [2]. Subsequently we used the recently developed rat $P2Y_2$ antibody from M. Knepper/B. Kishore and demonstrated specific staining also of the mouse $P2Y_2$ receptor in M-1 cells (*unpublished observations*). In our recent study this $P2Y_2$ receptor was pharmacologically identified in the luminal membrane of principal cells of isolated perfused mouse CCD tubules [4]. It needs, however, be borne in mind that the pharmacological evidence, with UTP and ATP being of similar potency, would also allow the $P2Y_4$ receptor to be a candidate [33]. Yet, no other evidence would indicate that the latter receptor is involved. It has been described that epithelial cells can express a variety of different P2Y and P2X receptors [36]. We have not conducted a comprehensive screening of other putatively important P2 receptors expressed in M-1 CCD cells.

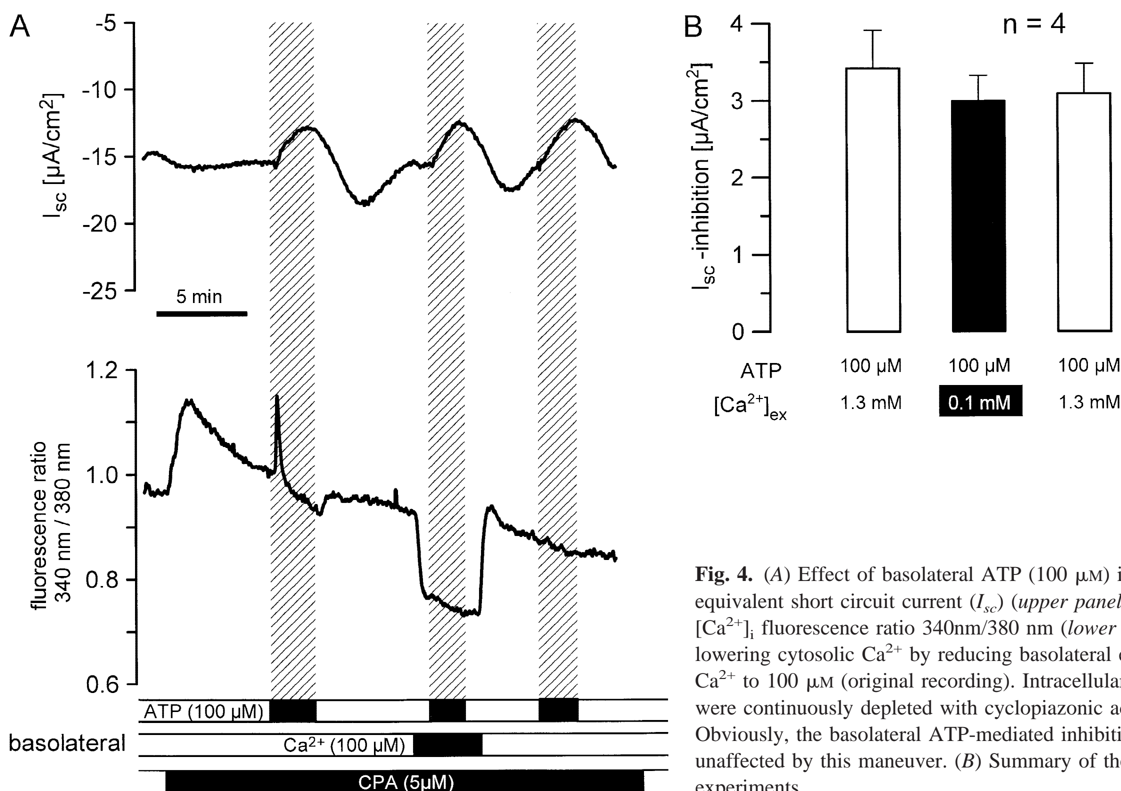

Fig. 4. (*A*) Effect of basolateral ATP (100 μM) in M-1 cells on equivalent short circuit current (I_{sc}) (*upper panel*) and Fura-2 $[Ca^{2+}]_i$ fluorescence ratio 340nm/380 nm (*lower panel*) after lowering cytosolic Ca^{2+} by reducing basolateral extracellular Ca^{2+} to 100 μM (original recording). Intracellular Ca^{2+} stores were continuously depleted with cyclopiazonic acid (5 μM). Obviously, the basolateral ATP-mediated inhibition of I_{sc} was unaffected by this maneuver. (*B*) Summary of the series of experiments.

$[Ca^{2+}]_i$, An Inhibitor of Electrogenic Na^+ Absorption in the Distal Nephron?

Over the past two decades numerous studies using microperfused collecting ducts have established that $[Ca^{2+}]_i$-elevating agonists like PGE_2 or acetylcholine inhibit Na^+ transport [13, 38]. Mostly rabbit collecting ducts have been used and numerous reports firmly support the involvement of a cytosolic $[Ca^{2+}]_i$ elevation mediating the inhibition of Na^+ transport [8, 13, 38]. Downstream of the $[Ca^{2+}]_i$ signal PKC activation has been suggested to mediate the inhibitory effect on Na^+ transport [3]. Thus, in rabbit tissue the numerous studies draw a homogeneous picture of these initial signaling events.

In contrast, in rodents intracellular Ca^{2+} and PKC do not inhibit electrogenic Na^+ absorption [34]. The present study demonstrates, that in the mouse the elevation of $[Ca^{2+}]_i$ does not appear to mediate an inhibition of Na^+ absorption. CPA initially increased $[Ca^{2+}]_i$, but never altered electrogenic Na^+ transport. This was also observed by others when thapsigargin was used to elevate $[Ca^{2+}]_i$ in M-1 cells [28]. A close inspection of Fig. 1 or 2 indicates that under continuous administration of CPA basolateral ATP decreases $[Ca^{2+}]_i$. This observation is reminiscent of a previous study from our group in intestinal epithelial cells, in which we suggested that this phenomenon reflects the activation of the plasma membrane Ca^{2+} ATPase and therewith Ca^{2+} efflux [39]. The notion that nucleotide-mediated inhibition of Na^+ transport was Ca^{2+}-independent was further supported by experiments in which we lowered cytosolic Ca^{2+} more aggressively. We previously demonstrated that in renal and other epithelial cells the store-operated Ca^{2+} entry pathway is exclusively located in the basolateral membrane [11]. Lowering extracellular Ca^{2+} on the basolateral side during activated store-operated Ca^{2+} entry markedly lowered cytosolic Ca^{2+}. This $[Ca^{2+}]_i$ lowering maneuver left the ATP-mediated inhibition of Na^+ transport unaffected. Thus, the presented data show that in mouse M-1 cells the nucleotide-induced inhibition of Na^+ absorption is regulated independently of $[Ca^{2+}]_i$. These data add to the picture describing Ca^{2+}-independent regulation of Na^+ transport in rodent cortical collecting duct tissue.

The effect of luminal and basolateral P2-receptor activation has recently also been investigated in cultured rabbit connecting tubule cells [18]. The authors could show that extracellular nucleotides via the $P2Y_2$ receptor decrease Na^+ and Ca^{2+} absorption. This was still true if cells were incubated with BAPTA-AM to buffer cytosolic Ca^{2+}. Although the $[Ca^{2+}]_i$ signal was not measured in the same experiments, these data suggest that also in rabbit distal tubular cells the ATP/UTP-mediated inhibition of Na^+ and Ca^{2+} absorption was regulated independently of an increase in cytosolic Ca^{2+}.

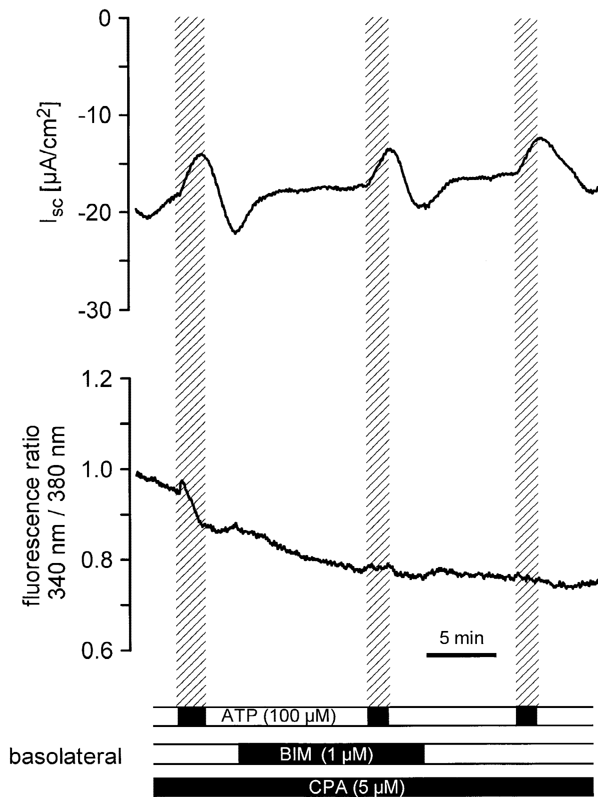

Fig. 5. Effect of the PKC inhibitor bisindolylmaleimide (BIM, 1 μM) on basolateral ATP (100 μM)-induced inhibition of I_{sc} in M-1 cells (original recording). *Upper trace:* equivalent short-circuit current (I_{sc}). *Lower trace:* Fura-2 $[Ca^{2+}]_i$ fluorescence ratio 340nm/380nm. Intracellular Ca^{2+} stores were continuously depleted with cyclopiazonic acid (5 μM). PKC inhibition with BIM did not affect the ATP-mediated inhibition of I_{sc}.

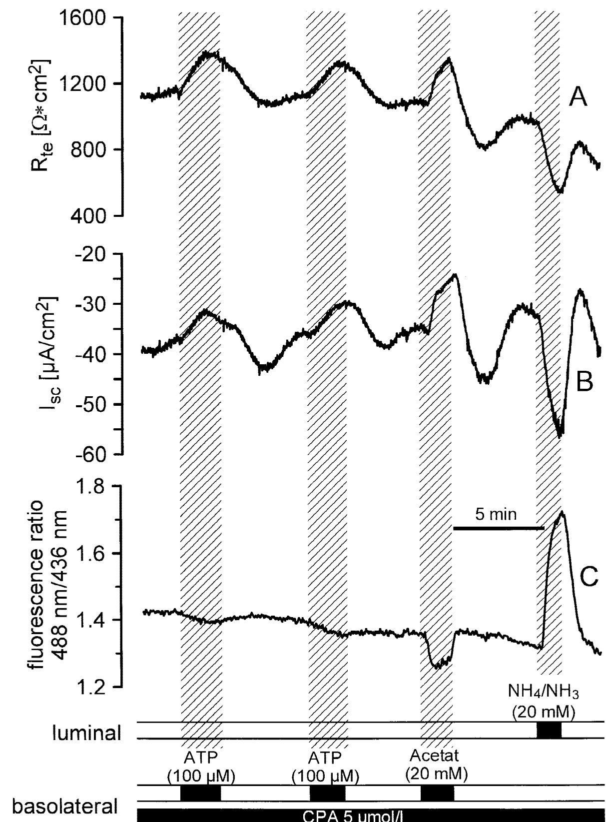

Fig. 6. Effect of basolateral ATP (100 μM) on intracellular pH (pH_i) in M-1 cells. Depicted are: *A,* transepithelial resistance (R_{te}); *B,* equivalent short-circuit current (I_{sc}); and *C,* BCECF pH_i-fluorescence ratio 488nm/436nm (original recording). Basolateral ATP induced the above described transepithelial electrical changes and small pH_i acidification. Also depicted are an acetate (20 mM)-induced acidification and the corresponding inhibition of I_{sc} and an NH_3/NH_4-stimulated alkalinization with its corresponding activation of I_{sc}.

Involvement of PKC

In subsequent experiments the involvement of PKC was investigated. PKC inhibitors like bisindolylmaleimide or staurosporine showed no effect. Here it is noteworthy that staurosporine at a concentration >0.5 μM led to an irreversible break-down of R_{te} and could thus not be used. Furthermore, the use of a broad-spectrum PKC activator like PMA did not mimic the effect of ATP/UTP. Thus, in M-1 cells the nucleotide-induced inhibition of Na^+ transport appears not to involve the activation of PKC. These results are similar to a study in perfused rat CCD [34].

In rabbit tissue, however, the involvement of PKC activation in agonist-mediated inhibition of Na^+ transport is clearly indicated. Essentially all studies addressing this question with different inhibitory transmitters like PGE_2, acetylcholine or nucleotides find an involvement of PKC [3, 18, 38]. More recent studies indicate that the PKC epsilon is the relevant kinase isoform in rabbit tissue [3].

Involvement of pH_i

Our experiments revealed that ATP, indeed, acidifies M-1 cells. Recent studies from other epithelia also indicate that P2-receptor activation induces an intracellular acidification [14, 19]. The mechanism involved in these ATP-mediated acidifications remains to be elucidated. For M-1 cells it thus appears possible that ATP-inhibited Na^+ transport occurs via a decrease in pH_i. However, this acidification was very small and thus can not be considered an important regulator of the ATP/UTP-mediated inhibition of Na^+ absorption. However, we feel safe to conclude that the ATP-mediated acidification contributes to a small extent to the observed phenomenon.

Mechanism of ATP-Mediated Inhibition of Na^+ Transport

We currently do not know either the critical transduction event involved in the ATP-mediated inhibition of Na^+

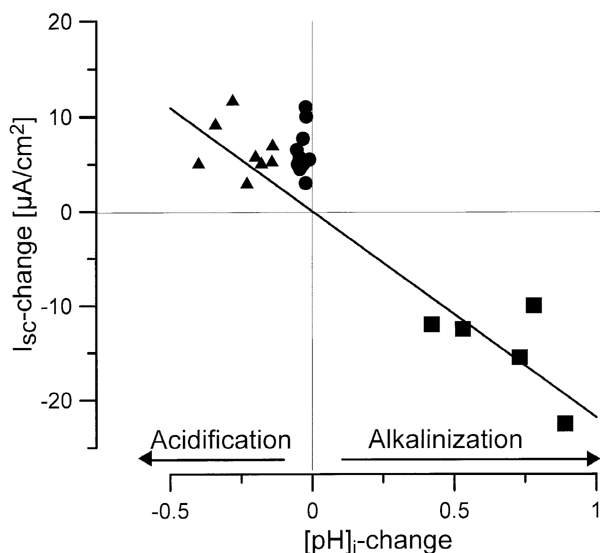

Fig. 7. Correlation analysis of pH_i alteration-mediated changes of I_{sc}. The ordinate shows the induced change of I_{sc}. The abscissa depicts the corresponding pH_i changes induced by the different maneuvers. Each symbol represents an individual experiment: ▲, a single 'acetate' experiment; ■, a single NH_4/NH_3 experiment; and ●, a single ATP experiment. In the range of ±0.5 pH_i units around resting pH_i a linear correlation was observed between the acetate-induced inhibition or the NH_3/NH_4-stimulated activation of I_{sc}. As is obvious, the ATP-induced effects on I_{sc} and pH_i do not superimpose onto this regression line.

absorption in M-1 cells or the regulated membrane transport molecule. In general, the parallel function of luminal ENaC channels, the basolateral Na^+/K^+ ATPase, and a basolateral K^+ conductance are required to allow the transcellular movement of Na^+. Thus, the inhibition of any of these transporting modules could account for the observed ATP effect. Here an interesting preliminary study in A6 cells suggests that luminal ATP via P2Y receptors inhibits ENaC channels [25]. Other studies in epithelial cells showed that P2-receptor activation leads to the inhibition of K^+ conductances [5, 14, 24]. It thus may appear that the inhibition of a basolateral K^+ conductance, which would reduce the basolateral recycling of K^+ and driving force for luminal Na^+ entry, could be another mechanism of the ATP-effect in Na^+-transporting kidney tubule cells. Using a method described by Yonath and Civan, the electromotive driving force (EMF) for transepithelial Na^+ transport can be quantified [40]. To this end we plotted the conductance versus the I_{sc}, where the inverse slope gives an estimate of the EMF. EMF was calculated before and during the ATP action and no difference could be observed (before ATP: 49.1 ± 7.9 mV; during ATP: 49.7 ± 4.2 mV, $n = 7$). It therefore appears unlikely that the inhibition of a basolateral K^+ conductance is involved in the ATP action.

In summary, we have investigated the detailed regulatory steps involved in the ATP/UTP-mediated inhibition of Na^+ transport. We identified that the ATP/UTP-mediated Na^+-transport inhibition 1) occurs independently of $[Ca^{2+}]_i$, 2) does not involve the activation of PKC and 3) is to a small part mediated through an intracellular acidification. Most probably the inhibited membrane transport molecule is the luminal epithelial Na^+ channel. In a preliminary study we recently demonstrated that also in intact, freshly isolated mouse CCD luminal and basolateral $P2Y_2$-receptor activation inhibits electrogenic Na^+ absorption [22].

The authors gratefully acknowledge the expert technical assistance by G. Kummer and B. Platz. Dr. U. Fröbe, Ing. H.J. Weber have been of greatest help in the construction and design of hard- and software used in these experiments. Especially the technical workshop mechanic H. Kressner has been of invaluable help in constructing the perfusion chambers. This study was supported by DFG, Le 942/4-1 and the German Academic Exchange Service.

References

1. Cha, S.H., Sekine, T., Endou, H. 1998. P2 purinoceptor localization along rat nephron and evidence suggesting existence of subtypes $P2Y_1$ and $P2Y_2$. *Am. J. Physiol.* **274:**F1006–F1014
2. Cuffe, J.E., Bielfeld-Ackermann, A., Thomas, J., Leipziger, J., Korbmacher, C. 2000. ATP stimulates Cl^- secretion and reduces amiloride-sensitive Na^+ absorption in M-1 mouse cortical collecting duct cells. *J. Physiol.* **524:**77–90
3. DeCoy, D.L., Snapper, J.R., Breyer, M.D. 1995. Anti-sense DNA down-regulates proteins kinase C-epsilon and enhances vasopressin-stimulated Na^+ absorption in rabbit cortical collecting duct. *J. Clin. Invest.* **95:**2749–2756
4. Deetjen, P., Thomas, J., Lehrmann, H., Kim, S.J., Leipziger, J. 2000. The luminal P2Y receptor in the isolated perfused mouse cortical collecting duct. *J. Am. Soc. Nephrol.* **11:**1798–1806
5. Devor, D.C., Pilewski, J.M. 1999. UTP inhibits Na^+ absorption in wild-type and DeltaF508 CFTR-expressing human bronchial epithelia. *Am. J. Physiol.* **276:**C827–C837
6. Ecelbarger, C.A., Maeda, Y., Gibson, C.C., Knepper, M.A. 1994. Extracellular ATP increases intracellular calcium in rat terminal collecting duct via a nucleotide receptor. *Am. J. Physiol.* **267:** F998–F1006
7. Frindt, G., Palmer, L.G., Windhager, E.E. 1996. Feedback regulation of Na channels in rat CCT. IV. Mediation by activation of protein kinase C. *Am. J. Physiol.* **270:**F371–F376
8. Frindt, G., Windhager, E.E. 1990. Ca^{2+}-dependent inhibition of sodium transport in rabbit cortical collecting tubules. *Am. J. Physiol.* **258:**F568–F582
9. Garty, H., Civan, E.D., Civan, M.M. 1985. Effects of internal and external pH on amiloride-blockable Na^+ transport across toad urinary bladder vesicles. *J. Membrane Biol.* **87:**67–75
10. Garty, H., Palmer, L.G. 1997. Epithelial Sodium Channels: Function, Structure, and Regulation. *Physiol. Rev.* **77:**359–451
11. Gordjani, N., Nitschke, R., Greger, R., Leipziger, J. 1997. Capacitative Ca^{2+} entry (CCE) induced by luminal and basolateral ATP in polarized MDCK-C7 cells is restricted to the basolateral membrane. *Cell Calcium* **22:**121–128
12. Guan, Y., Zhang, Y., Breyer, R.M., Fowler, B., Davis, L., Hebert, R.L., Breyer, M.D. 1998. Prostaglandin E2 inhibits renal collecting duct Na^+ absorption by activating the EP1 receptor. *J. Clin. Invest.* **102:**194–201
13. Hebert, R.L., Jacobson, H.R., Breyer, M.D. 1991. Prostaglandin E2

inhibits sodium transport in rabbit cortical collecting duct by increasing intracellular calcium. *J. Clin. Invest.* **87:**1992–1998

14. Hede, S.E., Amstrup, J., Christoffersen, B.C., Novak, I. 1999. Purinoceptors evoke different electrophysiological responses in pancreatic ducts. P2Y inhibits K^+ conductance, and P2X stimulates cation conductance. *J. Biol. Chem.* **274:**31784–31791
15. Kerstan, D., Gordjani, N., Nitschke, R., Greger, R., Leipziger, J. 1998. Luminal ATP induces K^+ secretion via a $P2Y_2$ receptor in rat distal colonic mucosa. *Pfluegers Arch. Eur. J. Physiol.* **436:**712–716
16. Kishore, B.K., Chou, C.-L., Knepper, M.A. 1995. Extracellular nucleotide receptor inhibits AVP-stimulated water permeability in inner medullary collecting duct. *Am. J. Physiol.* **269:**F863–F869
17. Kishore, B.K., Ginns, S.N., Krane, C.M., Nielsen, S., Knepper, M.A. 2000. Cellular localization of $P2Y_2$ purinoceptor in rat renal medulla and lung. *Am. J. Physiol.* **278:**F43–F51
18. Koster, H.P.G., Hartog, A., van Os, C.H., Bindels, R.J.M. 1996. Inhibition of Na^+ and Ca^{2+} reabsorption by P2U purinoceptors requires PKC but not Ca^{2+} signaling. *Am. J. Physiol.* **270:**F53–F60
19. Lee, M.G., Schultheis, P.J., Yan, M., Shull, G.E., Bookstein, C., Chang, E., Tse, M., Donowitz, M., Park, K., Muallem, S. 1998. Membrane-limited expression and regulation of Na^+-H^+ exchanger isoforms by P2 receptors in the rat submandibular gland duct. *J. Physiol.* **513:**341–357
20. Lee, M.G., Zeng, W., Muallem, S. 1997. Characterization and localization of P2 receptors in rat submandibular gland acinar and duct cells. *J. Biol. Chem.* **272:**32951–32955
21. Leipziger, J., Kerstan, D., Nitschke, R., Greger, R. 1997. ATP increases $[Ca^{2+}]_i$ and ion secretion via a basolateral P2Y receptor in rat distal colonic mucosa. *Pfluegers Arch. Eur. J. Physiol.* **434:**77–83
22. Leipziger, J., Lehrmann, H., Kim S.J., Thomas, J. 2000. Luminal $P2Y_2$-receptor mediated inhibition of Na^+ reabsorption in isolated perfused mouse CCD. *J. Am. Soc. Nephrol.* **11:**A173
23. Lohrmann, E., Burhoff, I., Nitschke, R.B., Lang, H.J., Mania, D., Englert, H.C., Hropot, M., Warth, R., Rohm, W., Bleich, M., Greger, R. 1995. A new class of inhibitors of cAMP-mediated Cl^- secretion in rabbit colon, acting by the reduction of cAMP-activated K^+ conductance. *Pfluegers Arch. Eur. J. Physiol.* **429:** 517–530
24. Lu, M., MacGregor, G.G., Wang, W., Giebisch, G.H. 2000. Extracellular ATP inhibits small-conductance K channels on the apical membrane of the cortical collecting duct from mouse kidney. *J. Gen. Physiol.* **116:**299–310
25. Ma, H.-P., Warnock, D.G. 2000. External ATP inhibits activation of ENaC in A6 cells by membrane stretch. *J. Am. Soc. Nephrol.* **11:**A178
26. McCoy, D.E., Taylor, A.L., Kudlow, B.A., Karlson, K., Slattery, M.J., Schwiebert, L.M., Schwiebert, E.M., Stanton, B.A. 1999. Nucleotides regulate NaCl transport in mIMCD-K2 cells via P2X and P2Y purinergic receptors. *Am. J. Physiol.* **277:**F552–F559
27. Morgan, A.J., Jacob, R. 1994. Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. *Biochem. J.* **300:**665–672
28. Nakhoul, N.L., Hering-Smith, K.S., Gambala, C.T., Hamm, L.L. 1998. Regulation of sodium transport in M-1 cells. *Am. J. Physiol.* **275:**F998–F1007
29. Nitschke, R., Fröbe, U., Greger, R. 1991. Antidiuretic hormone acts via V_1 receptors on intracellular calcium in the isolated perfused rabbit cortical thick ascending limb. *Pfluegers Arch. Eur. J. Physiol.* **417:**622–632
30. Palmer, L.G., Frindt, G. 1987. Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. *Am J Physiol* **253:**F333–F339
31. Pearce, D., Verrey, F., Chen, S.Y., Mastroberardino, L., Meijer, O.C., Wang, J., Bhargava, A. 2000. Role of SGK in mineralocorticoid-regulated sodium transport. *Kidney Int.* **57:**1283–1289
32. Perrotti, N., He, R.A., Phillips, S.A., Renfrew, H.C., Taylor, S.I. 2001. Activation of serum- and glucocorticoid-induced protein kinase (SGK) by cyclic AMP and insulin. *J. Biol. Chem.* **276:**9406–9412
33. Ralevic, V., Burnstock, G. 1998. Receptors for purines and pyrimidines. *Pharmacol Rev* **50:**413–492
34. Rouch, A.J., Chen, L., Kudo, L.H., Bell, P.D., Fowler, B.C., Corbitt, B.D., Schafer, J.A. 1993. Intracellular Ca^{2+} and PKC activation do not inhibit Na^+ and water transport in rat CCD. *Am. J. Physiol.* **265:**F569–F577
35. Rouse, D., Leite, M., Suki, W.N. 1994. ATP inhibits the hydrostatic effect of AVP in rabbit CCT: evidence for a nucleotide P2U receptor. *Am. J. Physiol.* **267:**F289–F295
36. Schwiebert, E.M., King, S.R., Peti-Peterdi, J., Bell, D., Guay-Woodford, L., Taylor, A.L. 1999. P2Y and P2X purinergic receptor expression and function in PKD epithelia. *J. Am. Soc. Nephrol.* **10:**A2148
37. Stoos, B.A., Naray-Fejes-Toth, A., Carretero, O.A., Ito, S., Fejes-Toth, G. 1991. Characterization of a mouse cortical collecting duct cell line. *Kidney Int.* **39:**1168–1175
38. Takeda, M., Yoshitomi, K., Taniguchi, J., Imai, M. 1994. Inhibition of amiloride-sensitive apical Na^+ conductance by acetylcholine in rabbit cortical collecting duct perfused in vitro. *J. Clin. Invest.* **93:**2649–2657
39. Wolff, T., Leipziger, J., Fischer, K.-G., Klär, B., Nitschke, R., Greger, R. 1993. Evidence for agonist-induced export of intracellular Ca^{2+} in epithelial cells. *Pfluegers Arch. Eur. J. Physiol.* **424:**423–430
40. Yonath, J., Civan, M.M. 1971. Determination of the driving force of the Na^+ pump in toad bladder by means of vasopressin. *J. Membr. Biol.* **5:**366–385