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# Inhibition of Na<sup>+</sup> and Ca<sup>2+</sup> reabsorption by $P_{2u}$ purinoceptors requires PKC but not Ca<sup>2+</sup> signaling

HENK P. G. KOSTER, ANITA HARTOG, CAREL H. VAN OS, AND RENÉ J. M. BINDELS Department of Cell Physiology, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

Koster, Henk P. G., Anita Hartog, Carel H. van Os, and René J. M. Bindels. Inhibition of Na<sup>+</sup> and Ca<sup>2+</sup> reabsorption by  $P_{2u}$  purinoceptors requires PKC but not Ca<sup>2+</sup> signaling. Am. J. Physiol. 270 (Renal Fluid Electrolyte Physiol. 39): F53–F60, 1996.—Rabbit connecting tubule and cortical collecting duct cells were isolated by immunodissection and cultured to confluence on permeable filters and on glass coverslips. Extracellular ATP dose-dependently reduced tran-

originally derived from kidneys of *Xenopus laevis* and exhibit a distal tubule phenotype. In these cells, ATP increased  $[Ca^{2+}]_i$  and stimulated  $Cl^+$  secretion and Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransport (18).

The aim of the present study was to examine the effect of extracellular ATP on transcellular Na<sup>+</sup> and Ca<sup>2+</sup> transport in the rabbit cortical collecting system and the signaling pathway involved in the cellular response to ATP. For this purpose, primary cultures of immunodissected rabbit cortical collecting system cells were used, which retain many characteristics of the original epithelium (2). This model system expresses an amiloride-sensitive lumen-negative transepithelial potential difference, and parathyroid hormone- and 1,25-dihydroxyvitamin D<sub>3</sub>-sensitive transcellular Ca<sup>2+</sup> transport (4).

scellular Na<sup>+</sup> and Ca<sup>2+</sup> transport (half-maximal inhibitory concentration, IC<sub>50</sub>, of 0.5  $\pm$  0.2 and 3.2  $\pm$  0.5  $\mu$ M), with a maximal inhibition of 57  $\pm$  5 and 43  $\pm$  4%, respectively. Purinergic receptor agonists inhibited transport with the following rank order of potency: UTP = ATP > ADP; this suggests involvement of  $P_{2u}$  purinoceptors. ATP also caused a dose-dependent (50% effective dose, EC<sub>50</sub>, of 1.5  $\pm$  0.2  $\mu$ M) transient increase in intracellular  $Ca^{2+}$  concentration ([ $Ca^{2+}]_i$ ), which decreased to a sustained elevated level. In the absence of extracellular  $Ca^{2+}$ , a similar  $Ca^{2+}$  transient occurred, but the sustained response was abolished. Preloading the cells with the  $Ca^{2+}$  chelator 1,2-bis(2-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid (BAPTA) completely prevented the ATP-induced  $Ca^{2+}$  transients, but not the ATP-induced inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption. Activation of protein kinase C (PKC) by the cell-permeable diacylglycerol analogue, 1,2-dioctanoyl-sn-glycerol, mimicked ATP-induced inhibition of Na<sup>+</sup> and Ca<sup>2+</sup> absorption. The inhibitory effects of ATP were no longer observed after culturing cells in the presence of phorbol ester (12-O-tetradecanoylphorbol-13-

#### MATERIALS AND METHODS

Primary cultures of cells from rabbit kidney cortical collecting system. Rabbit kidney connecting tubule and cortical collecting duct cells, hereafter referred to as cortical collecting system, were immunodissected with monoclonal antibody R2G9 and set in primary culture on permeable filters (0.3 cm<sup>2</sup>; Costar, Badhoevedorp, Netherlands) or on 22-mm circular glass coverslips, as previously described in detail (5). In brief, New Zealand White rabbits ( $\approx 0.5$  kg body wt) were used. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium (DME)-F12 medium (GIBCO, Breda, Netherlands) supplemented with 5% (vol/vol) decomplemented fetal calf serum, 50 µg/ml gentamicin, 10 µl/ml nonessential amino acids (GIBCO), 5 µg/ml insulin, 5 µg/ml transferrin, 50 nM hydrocortisone, 70 ng/ml prostaglandin  $E_1$ , 50 nM Na<sub>2</sub>SeO<sub>3</sub>, and 5 pM triiodothyronine, equilibrated with 5%  $CO_2$ -95% air at 37°C. All experiments were performed with confluent monolayers between 5 and 8 days after seeding the cells. Determination of transcellular short-circuit current. Filter cups were mounted between two half-chambers (area of 0.3) cm<sup>2</sup>) and bathed at 37°C with incubation medium containing (in mM) 140 NaCl, 2 KCl, 1 K<sub>2</sub>HPO<sub>4</sub>, 1 KH<sub>2</sub>PO<sub>4</sub>, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 5 glucose, 5 L-alanine, and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-tris(hydroxymethyl)aminomethane, pH 7.40 (unless otherwise indicated). The solutions bathing the monolayer were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA), and the shortcircuit current  $(I_{sc})$  was recorded. The benzamil-sensitive component of  $I_{sc}$  was used as an estimate of transcellular Na<sup>+</sup> transport. Determination of transcellular  $Ca^{2+}$  fluxes. Filter cups were washed and bathed at 37°C in incubation medium. Previously, we determined that transcellular  $Ca^{2+}$  absorption from a medium containing 1 mM  $Ca^{2+}$  was linear up to 3 h (6). In the present study,  $Ca^{2+}$  absorption was established by removing duplicate 25-µl aliquots of apical fluid after 90 min of incubation in the presence or absence of test drugs. The  $Ca^{2+}$ content of the samples was assayed using a colorimetric test

acetate) for 5 days, which resulted in downregulation of cellular PKC activity.

connecting tubule; cortical collecting duct; sodium channel; adenosine triphosphate; purinergic receptor; cortical collecting system

EXTRACELLULAR ATP HAS BEEN shown to play a significant role in many biological processes including neurotransmission, smooth muscle contraction and relaxation, modulation of ion channels, and cellular transport processes (10). ATP is released into the extracellular space by synaptic release from neurons, by release from cytoplasmic stores and in more pathological situations from lysed cells (10, 11). Concentrations of ATP reach sufficient levels to activate so-called  $P_2$  purinoceptors. In general, this class of purinoceptors couples to phospholipase C and produces both a rise in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) and activation of protein kinase C(PKC)(11). Although effects of extracellular ATP have been investigated in various cell types, relatively few studies are known of ATP actions on renal epithelial cells (14). P<sub>2</sub> purinoceptors have been shown to be present on established renal cell lines and on primary cultures of proximal tubules, suggesting that ATP is able to modulate ion transport across renal cells (1, 8, 18, 21). This was shown for the first time in A6 cells, which were

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kit (Boehringer, Mannheim, Germany), and Ca<sup>2+</sup> absorption was expressed in nanomoles per hour per square centimeter.

The time dependence of the inhibitory effect of ATP on  $Ca^{2+}$ transport was investigated by determining the apical-tobasolateral <sup>45</sup>Ca<sup>2+</sup> flux. The filter cups were washed twice with incubation medium containing 1 mM  $Ca^{2+}$  (37°C), and  $0.4 \ \mu Ci^{45}Ca^{2+}$  was added to the apical compartment. At 6-min intervals, 20-µl samples were taken from the basolateral compartment and counted for radioactivity.

Measurement of  $[Ca^{2+}]_i$  in single cells. The Ca<sup>2+</sup>-sensitive fluorescent dye, fura 2, was loaded into the cells during a 30-min incubation of the monolayer at 37°C in DME-F12 medium (GIBCO) supplemented with 5  $\mu$ M fura 2 acetoxymethyl ester (fura 2-AM), 0.4% (wt/vol) dimethyl sulfoxide (DMSO), 0.02% (wt/vol) pluronic F-127, and 4% (vol/vol) decomplemented fetal calf serum. Thereafter, cell cultures were washed twice with incubation medium. Subsequently, coverslips were transferred to a thermostated "Leiden" chamber mounted on the stage of an inverted Diaphot microscope (Nikon, Amsterdam, NL) and connected to a MagiCal System (Applied Imaging Systems, UK), in which emitted light is captured with a charge-coupled device camera followed by digital imaging using TARDIS software (15). The cells were continuously superfused (2 ml/min) with medium, and the temperature was maintained at 37°C. The fluorescence of several adjacent cells emitted at 492 nm was collected at 0.5-s intervals at the excitation wavelengths 340 and 380 nm.  $[Ca^{2+}]_i$  was calculated from calibrated 340 nm/380 nm ratios corrected for background fluorescence as previously described in detail (15). The monolayers were exposed to 0.5% (wt/vol) fluorescein isothiocyanate-conjugated peanut lectin during the fura 2-AM loading procedure, allowing identification of type B intercalated cells before starting  $[Ca^{2+}]_i$  measurements (4). The majority of cells ( $\sim 60\%$ ) were peanut lectin negative and therefore identified as principal cells derived from either connecting tubule or cortical collecting duct. The responses of a large number of peanut lectin-negative cells were analyzed and averaged. Experimental protocols. ATP and other nucleotides were dissolved in water, whereas 12-O-tetradecanoylphorbol-13acetate (TPA), 1,2-dioctanoyl-sn-glycerol, fura 2-AM, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-AM were dissolved in DMSO. Final vehicle concentrations never exceeded 0.1% (vol/vol). Ca<sup>2+</sup>-free medium consisted of incubation medium without  $CaCl_2$ , but with 0.5 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-N, N, N', N'-

tetraacetic acid (EGTA). Results from experiments with vehicle alone were never significantly different from the control.

Materials. Collagenase A and hyaluronidase were obtained from Boehringer. Fura 2-AM, BAPTA-AM, and pluronic F-127 were purchased from Molecular Probes (Eugene, OR). <sup>45</sup>CaCl<sub>2</sub> was purchased from Amersham ('s-Hertogenbosch, Netherlands). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Statistical analysis. In all experiments, data were assessed from at least three separate isolations and expressed as the means  $\pm$  SE for *n* number of experiments. Statistical differences between the mean values were determined by analysis of variance (24). Unpaired Student's t-tests were used to determine statistical differences between two independent groups.

### RESULTS

Effect of extracellular ATP on Na<sup>+</sup> and Ca<sup>2+</sup> absorption. In Fig. 1A, it is shown that  $10^{-4}$  M ATP added to the apical or to the basolateral side of the monolayers significantly inhibited benzamil-sensitive  $I_{sc}$  by 56  $\pm$  8 and 44  $\pm$  15%, respectively (P < 0.05). ATP on both sides further increased inhibition of benzamil-sensitive  $I_{\rm sc}$  to 80  $\pm$  5% (P < 0.05). In addition, ATP inhibited Ca<sup>2+</sup> absorption by 11  $\pm$  3 and 15  $\pm$  2% when added to the apical or to the basolateral side, respectively (Fig. 1B). The inhibitory effects on  $Ca^{2+}$  absorption were additive, since addition of ATP to both compartments reduced Ca<sup>2+</sup> absorption by 28  $\pm$  2% (P < 0.05). In all following experiments, only apical effects of ATP or related nucleotides on benzamil-sensitive  $I_{\rm sc}$  were studied. Since the inhibitory action of ATP on  $Ca^{2+}$  absorption was much smaller, nucleotides were applied to both compartments in  $Ca^{2+}$  absorption assays. The short-term effects of ATP on benzamil-sensitive  $I_{\rm sc}$  and  ${\rm Ca^{2+}}$  absorption were studied, as presented in Fig. 2. Transepithelial Na<sup>+</sup> transport was immediately inhibited after addition of ATP, and a maximal inhibition was apparent after 10 min. Similarly, apical-tobasolateral  ${}^{45}Ca^{2+}$  flux was significantly reduced within 15 min after application.





Fig. 1. Effect of extracellular ATP on benzamil-sensitive short-circuit current ( $I_{sc}$ ; A) and Ca<sup>2+</sup> absorption (B) in rabbit cortical collecting system in primary culture. ATP was applied to the apical, basolateral, or both sides at a concentration of  $10^{-4}$  M. Values are means  $\pm$  SE (n = 15). \* Significantly different from control (P < 0.05).



Time (min)

Time (min)

Fig. 2. Time-dependent effect of extracellular ATP on benzamil-sensitive  $I_{sc}$  (A) and  ${}^{45}Ca^{2+}$  flux from apical-tobasolateral compartment (B). For A, at  $t = 5 \min$ , ATP ( $10^{-4}$  M) was added to apical compartment. For B, at  $t = 32 \min$ , ATP ( $\blacktriangle$ ,  $10^{-4}$  M) or vehicle ( $\bullet$ ) was added to both compartments. Representative traces of 4 experiments are shown.

In Fig. 3, the dose-response curves of ATP-induced inhibition of benzamil-sensitive  $I_{\rm sc}$  and  $Ca^{2+}$  absorption are shown. In both cases a maximal reduction was achieved at  $10^{-4}$  M ATP, and the half-maximal inhibitory concentration (IC<sub>50</sub>) was 0.5 ± 0.2 and 3.2 ± 0.5 µM, respectively.

To characterize the ATP receptor class mediating the inhibitory response to ATP, effects of other nucleotides on Na<sup>+</sup> and Ca<sup>2+</sup> absorption were investigated. Figure 4 demonstrates that UTP and ATP (both 10<sup>-4</sup> M) reduced benzamil-sensitive  $I_{sc}$  and Ca<sup>2+</sup> absorption with a similar potency, whereas ADP was ineffective (P > 0.2). Also AMP and adenosine did not mimic the inhibitory effect of ATP on Na<sup>+</sup> and Ca<sup>2+</sup> absorption (data not shown). Furthermore, the dose-response curve of UTP-induced inhibition of benzamil-sensitive  $I_{sc}$  and Ca<sup>2+</sup> absorption yielded an IC<sub>50</sub> of 1.7 ± 0.1 and 2.5 ± 0.2 µM, respectively (Fig. 5). These values are similar to the obtained IC<sub>50</sub> values for ATP. Taken together, this rank order of potency suggests that ATP exerts its inhibitory action on Na<sup>+</sup> and Ca<sup>2+</sup> absorption through a  $P_2$ -type of purinoceptor (10). Furthermore, these observations suggest that degradation of ATP by ectonucleotidases resulting in the formation of ADP, AMP, and adenosine is not responsible for the observed inhibition.

Effect of extracellular ATP on  $[Ca^{2+}]_i$ . Extracellular ATP (10<sup>-4</sup> M) induced a transient increase in  $[Ca^{2+}]_i$  in individual cells of the cortical collecting system, as depicted in Fig. 6. Within seconds after application of ATP, there is a fast rise in  $[Ca^{2+}]_i$  from resting levels to maximal values above 200 nM. After reaching a peak,  $[Ca^{2+}]_i$  returned to a sustained level, which was within

 $[Ca^{2+}]_i$  returned to a sustained level, which was within a few minutes slightly above the initial value, even in the continuous presence of ATP. Subsequent removal of extracellular Ca<sup>2+</sup> decreased this plateau to values not distinguishable from background levels. In Ca<sup>2+</sup>-free medium, the ATP-induced transient response leveled down to a sustained level, which was below the initial value, but increased again upon reapplication of Ca<sup>2+</sup>



Fig. 3. Dose-response curve of extracellular ATP on benzamil-sensitive  $I_{sc}(A)$  and  $Ca^{2+}$  absorption (B) in rabbit cortical collecting system in primary culture. Values are represented as percentage of maximal inhibition. Half-maximal inhibitory concentration (IC<sub>50</sub>) values of  $0.5 \pm 0.2 \mu M$  (A) and  $3.2 \pm 0.5 \mu M$  (B) were calculated. Values are means  $\pm$  SE (n = 9).





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Fig. 4. Effect of extracellular nucleotides  $(10^{-4} \text{ M})$  on benzamil-sensitive  $I_{sc}(A)$  and  $Ca^{2+}$  absorption (B) in rabbit cortical collecting system in primary culture. Values are means  $\pm$  SE (n = 9). \*Significantly different from control (P < 0.05).



Fig. 5. Dose-response curve of extracellular UTP on benzamil-sensitive  $I_{sc}(A)$  and  $Ca^{2+}$  absorption (B) in rabbit cortical collecting system in primary culture. Values are represented as percentage of maximal inhibition. IC<sub>50</sub> values of  $1.7 \pm 0.1 \mu M$  (A) and  $2.5 \pm 0.2 \mu M$  (B) were calculated. Values are means  $\pm SE$  (n = 2).



Fig. 6. Effect of extracellular ATP on intracellular  $Ca^{2+}$  concentration  $([Ca^{2+}]_i)$  in primary cultures of rabbit cortical collecting system cells in presence and absence of extracellular  $Ca^{2+}$   $([Ca^{2+}]_o)$ . A: response to  $10^{-4}$  M ATP in  $Ca^{2+}$ -containing medium and subsequent removal of extracellular  $Ca^{2+}$  by addition of 2 mM EGTA ( $[Ca^{2+}]_o = 0$  mM). B: response to  $10^{-4}$  M ATP in  $Ca^{2+}$ -free medium containing 0.5 mM EGTA and subsequent addition of 2 mM CaCl<sub>2</sub>. Representative traces of 5 experiments are shown.



Table 1. Effect of extracellular nucleotides on  $[Ca^{2+}]_i$ in primary cultures of rabbit cortical collecting system cells

	$\Delta[Ca^{2+}]_i$ , nM		
	Peak	Plateau	
UTP	134 - 12	20 mt 2	
ATP	$172 \pm 12$	20 million 33	
ADP	154 - 30	Terret in 15th Aguilde	

Values are means  $\pm$  SE; n = 20. All nucleotides were added at 10<sup>-4</sup> M.  $[Ca^{2+}]_i$ , intracellular  $Ca^{2+}$  concentration. The nucleotide-induced peak and plateau levels of  $[Ca^{2+}]_i$  are represented.

PKC activation or both. Experiments were performed to delineate the signal transduction pathway involved in inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption.

# Log [ATP] (M)

Fig. 7. Dose-response curve of extracellular ATP-mediated  $[Ca^{2+}]_i$ increase in primary cultures of rabbit cortical collecting system cells. Stimulations were carried out in fura 2-loaded cells in incubation medium supplemented with ATP concentrations as indicated. Values are represented as percentage of maximal response. A 50% effective dose (EC<sub>50</sub>) of 1.5  $\pm$  0.2  $\mu$ M was calculated. Values are means  $\pm$  SE (n - 16).

(Fig. 6B). In the absence of extracellular  $Ca^{2+}$  there was a small decrease in the peak height (197  $\pm$  17 vs. 264  $\pm$ 17 nM in presence of  $Ca^{2+}$ ; P < 0.05). These data suggest that ATP-stimulated increase of  $[Ca^{2+}]_i$  involves both  $Ca^{2+}$  release from intracellular stores and  $Ca^{2+}$  influx from extracellular medium.

To corroborate further the effect of extracellular ATP on  $[Ca^{2+}]_i$ , a dose-response curve of the ATP-induced increase in  $[Ca^{2+}]_i$  was obtained, as shown in Fig. 7. A maximal effect was found at ATP concentrations around  $10^{-4}$  M. An 50% effective dose (EC<sub>50</sub>) of 1.5 ± 0.2 µM was calculated, which is of similar magnitude to the  $IC_{50}$  value obtained for the ATP-induced inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption. Figure 8 shows a representative experiment, in which the responses to UTP and ADP obtained in a single cell of the cortical collecting system were compared with the effect of ATP. All three nucleotides increased  $[Ca^{2+}]_i$ transiently, which was followed by a sustained phase. The nucleotide-induced maximal and plateau values are summarized in Table 1. These findings confirm the conclusion that ATP activates  $P_2$  purinoceptors in primary cultures of the cortical collecting system. Signal transduction pathways in Na<sup>+</sup> and  $Ca^{2+}$ ated by PKC activation. absorption inhibition. The ATP-induced increase in  $[Ca^{2+}]_i$  suggests P<sub>2</sub>-purinoceptor activation of phospho-DISCUSSION lipase C, resulting in inositol trisphosphate ( $IP_3$ ) and The present study demonstrates that extracellular diacylglycerol (DAG) (10). In principle, the inhibitory actions of ATP may be mediated by either  $[Ca^{2+}]_i$  or ATP inhibits Na<sup>+</sup> and  $Ca^{2+}$  absorption in rabbit cortical

Therefore, cells of the cortical collecting system were loaded with the  $Ca^{2+}$  chelator BAPTA to prevent the ATP-induced increase in  $[Ca^{2+}]_i$ . As shown in Fig. 9A, preloading the monolayers for 5 min with 15  $\mu$ M BAPTA-AM abolished the ATP-induced  $[Ca^{2+}]_i$  response, but this maneuver failed to reduce the ATPinduced inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption (Fig. 9B, P > 0.2). This result provides strong evidence that the  $[Ca^{2+}]_i$  pathway is not mediating the ATP-induced transport inhibitions.

In a previous study, we reported that pharmacological activation of PKC by TPA had a dual effect on Na<sup>+</sup> and  $Ca^{2+}$  absorption (3). Short-term exposure (e.g., minutes) to TPA increased PKC activity and decreased Na<sup>+</sup> and Ca<sup>2+</sup> absorption, whereas long-term exposure (e.g., days) resulted in downregulation of total cellular PKC activity with a complete recovery of transcellular Na<sup>+</sup> and  $Ca^{2+}$  transport rate. In the present study, 1,2-dioctanoyl-sn-glycerol (10<sup>-4</sup> M), a cell-permeable DAG analogue, essentially mimicked the inhibitory effect of ATP on Na<sup>+</sup> and  $Ca^{2+}$  absorption, as shown in Fig. 8. Furthermore, cells of the cortical collecting system were cultured in the presence of 10<sup>-6</sup> M TPA for 5 days to downregulate PKC activity, and subsequently the effect of ATP on Na<sup>+</sup> and  $Ca^{2+}$  absorption was determined (Fig. 10). ATP (10<sup>-4</sup> M) was no longer able to inhibit Na<sup>+</sup> and Ca<sup>2+</sup> absorption when cells were cultured in the presence of TPA (P > 0.2). Taken together, these results strongly suggest that ATPinduced inhibition of Na<sup>+</sup> and Ca<sup>2+</sup> absorption is medi-



Fig. 8. Effect of extracellular nucleotides (10 4 M) on [Ca<sup>2+</sup>], in primary cultures of rabbit cortical collecting system cells. Representative traces of 4 experiments are shown.



Fig. 9. Effect of 1,2-bis(2-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid (BAPTA) loading on ATP-induced increase of  $[Ca^{2+}]_i$  (A) and inhibition of benzamil-sensitive  $I_{sc}$  and  $Ca^{2+}$  absorption (B) in primary cultures of rabbit cortical collecting system cells. At time 0, acetoxymethyl ester of BAPTA was added to the medium at a final concentration of 15  $\mu$ M. ATP was added to the medium at a final concentration of 10<sup>-4</sup> M. Values are means  $\pm$  SE (n = 15).

collecting system in primary culture via activation of a  $P_{2u}$  purinoceptor coupled to phospholipase C, which produces both a rise in  $[Ca^{2+}]_i$  and activation of PKC. The inhibitory actions of ATP are mediated by the  $P_2$ -receptor subtypes like  $P_{2x}$ ,  $P_{2y}$ ,  $P_{2z}$ , and  $P_{2t}$ , it PKC-dependent pathway, whereas the  $Ca^{2+}$ -dependent route was not essential.

Extracellular ATP is known to induce a variety of responses by acting on purinergic receptors in several cell types (10, 11). Purinergic receptors have been classified into two groups on the basis of agonist selectivity, i.e., with ATP and UTP preferring P<sub>2</sub> purinoceptors and with adenosine preferring  $P_1$  purinoceptors. In the present study, the rank order of potency for absorption. ATP, UTP and ADP initiated a comparable inhibition of Na<sup>+</sup> and Ca<sup>2+</sup> absorption was UTP = increase in  $[Ca^{2+}]_i$ . For instance, extracellular ATP

ATP > ADP, which suggests that the inhibitory effect of ATP is mediated by  $P_2$  purinoceptors. Since previous studies have demonstrated that UTP has little effect on appears that in our model system the purinoceptor activated by ATP is the nucleotide subtype, i.e.,  $P_{2u}$  (10). The effect of ATP was further characterized by a dose-dependent inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption with an  $IC_{50}$  value in the micromolar range and a maximal inhibition of  $\sim 50\%$ .

We have addressed the question which signaling pathway is involved in inhibition of Na<sup>+</sup> and  $Ca^{2+}$ 



Fig. 10. ATP-induced inhibition of benzamil-sensitive  $I_{sc}$  and  $Ca^{2+}$  absorption in rabbit cortical collecting system in primary culture is mediated by protein kinase C. Cells were cultured in the presence of  $10^{-6}$  M 12-Otetradecanoylphorbol-13-acetate (TPA) for 5 days to downregulate protein kinase C (PKC) as described previously (3). Control cells (control) were cultured during the same period in presence of solvent only. Subsequently, the PKC-downregulated and control cells were exposed to 10<sup>-4</sup> MATP. In addition, 10<sup>-4</sup> M of 1,2-dioctanoyl-sn-glycerol ("DAG"), a cell-permeable diacylglycerol analogue, was added to control cells. Values are means  $\pm$  SE (n = 9). \*Significantly different from control (P < 0.05).

evoked a transient rise in  $[Ca^{2+}]_i$  that is primarily due to release of  $Ca^{2+}$  from internal stores. Agonist interaction with P<sub>2</sub> purinoceptors has frequently been shown to activate also plasmalemmal  $Ca^{2+}$  channels (10, 11). This was indeed the case in rabbit cortical collecting system cells, since the sustained phase was totally dependent on extracellular  $Ca^{2+}$ . Thus our data are consistent with an ATP receptor that activates phospholipase C, resulting in IP<sub>3</sub> and DAG, as has been shown in a variety of cells (10, 19).

The dose dependency of the ATP-induced increase in  $[Ca^{2+}]_i$  and subsequent functional effects displayed a similar potency, which suggests a causal relationship between  $[Ca^{2+}]_i$  and the inhibition of Na<sup>+</sup> and Ca<sup>2+</sup>

Ca<sup>2+</sup> absorption. Second, downregulation of PKC activity completely abolished the inhibitory effect of ATP. These findings strongly imply that PKC activation is responsible for the ATP-induced inhibition of Na<sup>++</sup> and  $Ca^{2+}$  absorption in the rabbit cortical collecting system. At present it is known that PKC exists as a family of at least 12 isozymes, all having closely related structures but differing in tissue expression and in substrate specificity (9). Most PKC isozymes require micromolar  $[Ca^{2+}]_i$  and DAG for activation, while some, including  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ , and  $\zeta$ , exhibit  $Ca^{2+}$ -independent activation. To date three isozymes,  $\alpha$ ,  $\beta$ , and  $\zeta$ , have been detected in the kidney (7). Our data imply that a  $Ca^{2+}$ -independent subtype of PKC is involved in the actions of ATP. Interestingly, in rat renal mesangial cells PKC- $\epsilon$  has been shown to mediate the cellular response induced by extracellular ATP and UTP (22). Further biochemical studies, however, are needed to identify the hormonally activated PKC isozymes in the cortical collecting system. The present study adds physiological relevance to the previously demonstrated PKC-mediated inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption in the cortical collecting system. Since the kidney is a particularly rich source of ATP and UTP, it is intriguing to consider the presence of  $P_2$  purinoceptors as a means of regulation of renal ion transport processes (18). ATP can accumulate in the extracellular space either during normal metabolism or in pathological conditions by release from several cell types present in the kidney, including neuronal, endothelial, and smooth muscle cells (14). Locally, ATP concentrations may amount to quantities in the micromolar range, exceeding the concentrations required to evoke cellular responses (14). Studies in cultured renal epithelial cells, such as proximal tubules, cortical collecting system, LLC-PK<sub>1</sub>, MDCK, and A6 cells, illustrate the presence of functional ATP receptors and hence the potential to modulate electrolyte and water transport (present study, 1, 8, 18, 21). The existence of a  $P_{2n}$ receptor in the kidney has been shown recently (17). The cloning and expression of a  $P_{2u}$  receptor in neuroblastoma cells were reported, and Northern blot analysis revealed that this receptor is expressed in the kidney (17). In addition, Rouse et al. (23) demonstrated that extracellular ATP via activation of a  $P_{2u}$ -receptor subtype inhibits the hydrosmotic effect of arginine vasopressin in isolated perfused rabbit cortical collecting ducts. In conclusion, our study provides evidence for the presence of ATP receptors in rabbit cortical collecting system in primary culture and demonstrates its potential role in modulating ion transport processes in this nephron segment. Binding of ATP to  $P_{2u}$  purinoceptors, activation of phospholipase C, and a  $Ca^{2+}$ -independent PKC isozyme are the most likely biochemical events accumulating in inhibition of Na<sup>+</sup> and  $Ca^{2+}$  reabsorption. The target or substrate for PKC may be either apical Na<sup>+</sup> and Ca<sup>2+</sup> channels or basolateral Na<sup>+</sup>-K<sup>+</sup>adenosinetriphosphatase (Na+-K+-ATPase), Ca<sup>2+</sup>-ATPase, or Na<sup>+</sup>/Ca<sup>2+</sup> exchange. However, elucidation of

absorption. However, when cells were loaded with the  $Ca^{2+}$  chelator BAPTA to quench increases in  $[Ca^{2+}]_i$ , the ATP-induced inhibition of Na<sup>+</sup> and  $Ca^{2+}$  absorption remained essentially unaltered, which strongly suggests that there is no functional relationship between these two ATP-induced processes. This finding is remarkable in view of the consensus that an increase in  $[Ca^{2+}]_i$  is an important signal transduction pathway in cellular responses to hormones. For instance, there is considerable evidence that transepithelial Na<sup>+</sup> transport is regulated in part by changes in  $[Ca^{2+}]_i$  that directly affect the apical Na<sup>+</sup> channel (13). In this respect it is of interest that Palmer and Frindt (20) suggested that the inhibitory effect of increased  $[Ca^{2+}]_i$ on Na<sup>+</sup> reabsorption in the cortical collecting duct was indirect and dependent on some additional effector (7, 20). Likewise, for transcellular  $Ca^{2+}$  transport, it has been proposed that  $[Ca^{2+}]_i$  plays a role in a negative feedback mechanism of apical  $Ca^{2+}$  entry (12). However, from our data it is clear that ATP exerts its inhibitory action on Na<sup>+</sup> and  $Ca^{2+}$  absorption via a pathway other than the  $[Ca^{2+}]_i$  transients. ADP increased  $[Ca^{2+}]_i$  with equipotency to ATP and UTP but had no detectable effect on Na<sup>+</sup> and Ca<sup>2+</sup> transport. This further suggests that the effect of ATP on  $[Ca^{2+}]_i$  and ion transport can be dissociated and implies that ADP and ATP activate distinct purinergic receptor subtypes. In diverse cell types, it has been shown that ATP- and ADP-induced increases in  $[Ca^{2+}]_i$ are mediated by several distinct mechanisms of action (10). Thus it is generally difficult to relate a nucleotideinduced increase in  $[Ca^{2+}]_i$  by activation of a purinergic receptor to its physiological role (11). Previously, we have demonstrated that the phorbol ester TPA inhibits Na<sup>+</sup> and Ca<sup>2+</sup> absorption in primary cultures of the rabbit cortical collecting system (3). In other studies, it was also shown that exogenous diacylglycerols and phorbol esters inhibit Na<sup>+</sup> absorption and  $K^+$  secretion in the collecting duct (7). Cell-attached patch-clamp studies suggest that PKC activation decreases Na<sup>+</sup> channel activity in the apical membrane of rabbit collecting ducts in primary culture (16). In our present study, these findings are further substantiated by two independent lines of evidence. First, the cellpermeable DAG analogue, 1,2-dioctanoyl-sn-glycerol, fully mimicked the ATP-induced inhibition of Na<sup>+</sup> and

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these targets requires biochemical studies, which were not feasible in the past but become now a realistic option, since we have shown that primary cultures of rabbit cortical collecting system cells provide a physiological alternative for isolated perfused tubules.

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Address for reprint requests: R. J. M. Bindels, 162 Cell Physiology, Univ. of Nijmegen, P.O.Box 9101, 6500 HB Nijmegen, the Netherlands.

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