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Purinergic Modulation of Na^+,K^+,Cl^- Cotransport and MAP Kinases is Limited to C11-MDCK Cells Resembling Intercalated Cells from Collecting Ducts

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Abstract. We demonstrated recently that in renal epithelial cells from collecting ducts of Madin-Darby canine kidneys (MDCK), Na^+,K^+,Cl^- cotransport is inhibited up to 50% by ATP via its interaction with P_{2Y} purinoceptors (*Biochim. Biophys. Acta* 1998. **1369:**233–239). In the present study we examined which type of renal epithelial cells possesses the highest sensitivity of Na^+,K^+,Cl^- cotransport to purinergic regulation. We did not observe any effect of ATP on Na^+,K^+,Cl^- cotransport in renal epithelial cells from proximal and distal tubules, whereas in renal epithelial cells from rabbit and rat collecting ducts ATP decreased the carrier's activity by ~30%. ATP did not affect Na^+,K^+,Cl^- cotransport in C7 subtype MDCK cells possessing the properties of principal cells but led to ~85% inhibition of this carrier in C11-MDCK cells in which intercalated cells are highly abundant. Both C7- and C11-MDCK exhibited ATP-induced IP_3 and cAMP production and transient elevation of $[Ca^{2+}]_i$. In contrast to the above-listed signaling systems, ATP-induced phosphorylation of ERK and JNK MAP kinases was observed in C11-MDCK only. Thus, our results reveal that regulation of renal Na^+,K^+,Cl^- cotransport by P_{2Y} receptors is limited to intercalated cells from collecting ducts and indicate the involvement of the MAP kinase cascade in purinergic control of this ion carrier's activity.

Key words: Na^+,K^+,Cl^- cotransport — P_2-purinoceptors — MAP kinases — Renal epithelium

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Introduction

As in most other tissues (Brake & Julius, 1996), ATP is co-released with norepinephrine from isolated kidneys upon renal sympathetic nerve stimulation (Bohmann et al., 1995). These results as well as data on the expression of mRNA encoding P_{2X1}, P_{2X4} (Takeda, Kobayashi & Endou, 1998; Chan et al., 1998) and 3 subtypes of P_{2Y} receptors (P_{2Y1}, P_{2Y2}, P_{2Y11}) (Rice, Burton & Fiedelday, 1995; Tokuyama et al., 1995; Webb et al., 1996; Communi et al., 1997; Takeda et al., 1998) in different segments of tubules and in cultured renal epithelial cells (REC) indicate that P_2 receptors are involved in the regulation of renal function. However, in contrast to nonepithelial cells, data on the role of P_2 receptors in the control of ion transporters contributing to reabsorption of salt and osmotically obliged water along the nephron are limited to a few publications. In monolayers of REC derived from the Madin-Darby canine kidney (MDCK), basolateral application of ATP leads to acute and sustained stimulation of short-circuit current resulting from basal to apical Cl^- secretion (Simmons, 1981), which seems to be mediated by two different types of P_{2Y} receptors (Zegarra-Moran, Rommeo & Galietta, 1995). Based on findings of P_{2Y} receptor-induced $[Ca^{2+}]_i$ elevation in REC, and in MDCK cells in particular (Paulmichl et al., 1991; Delles, Heller & Dietl, 1995), it has been suggested that the transient phase of ATP-induced increment of Cl^- secretion is caused by activation of Ca^{2+}-sensitive K^+-channels and modulation of membrane potential (Friedrich et al., 1989; Paulmichl et al., 1991; Tauc, Gastineau & Ponjeol, 1993). The role of other monovalent ion transporters in purinergic modulation of renal epithelium function remains unknown.

We reported earlier that in MDCK cells, Na^+,K^+,Cl^- cotransport is insensitive to agonists of P_1-purinergic, α-adrenergic, cholinergic and dopaminergic receptors as well as to vasopressin, bradykinin, angiotensin II and 8-Br-cGMP, but is completely blocked by activation of protein kinase C (PKC) with 4β-phorbol 12-myristate 13-acetate (PMA) and is partially inhibited by extracellular ATP (Gagnon et al., 1998). Recently, we demonstrated that ATP-induced inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells is triggered by P_{2Y} purinoceptors (Gagnon et al., 1999*a*). The present study examines (i) whether purinergic inhibition of Na^+,K^+,Cl^- cotransport is a universal property of REC or this regulatory pathway is a feature of collecting duct (CD)-derived MDCK, and (ii) which type of CD cells (principal or intercalated) possesses the highest Na^+,K^+,Cl^- cotransport sensitivity to purinergic regulation. To answer these questions, we compared the effect of ATP on K^+ (^{86}Rb) influx in MDCK cells, REC from rabbit proximal tubules (PT), distal tubules (DT) and CD, in REC from rat inner medullary CD as well as in C7 and C11 substrains of MDCK cells, in which principal and intercalated cells are highly abundant. The impact of ATP on $[Ca^{2+}]_i$, intracellular content of cAMP and inositol 1,4,5-triphosphate (IP_3) and MAP kinase phosphorylation was used as a positive control for the presence of P_{2Y} receptors and for analysis of the relative contributions of these signaling systems in purinergic inhibition of Na^+,K^+,Cl^- cotransport.

Materials and Methods

Cell Isolation and Culture

Epithelial cells from PT, DT and CD of New Zealand white rabbit kidneys were isolated and characterized according to a previously published procedure (Koop et al., 1991; Romero et al., 1992). These cells were seeded in medium containing DMEM-F12 powder, 15 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), 7 mM $NaHCO_3$, 350 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μM hydrocortisone, 5 μg/ml transferrin and 5% fetal calf serum and grown in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2–3 days and cells at passages 2 to 4 were used in this study. Cells from rat inner medullary CD (rIMCD) were obtained as described previously (Selvaggio et al., 1988) and used at passages 15 to 25. MDCK cells were procured from the American Type Culture Collection (ATCC No. CCL 34, Rockville, MD). Two subclones of MDCK cells possessing the properties of principal and intercalated cells from CD (C7-MDCK and C11-MDCK, respectively) were obtained as described previously in detail (Gekle et al., 1994). C7-MDCK and C11-MDCK cells seeded on permeable support have transepithelial resistance of 4905 ± 344 ($n = 3$) and 322 ± 32 Ω/cm^2 ($n = 10$), respectively, that is in accordance with previously reported data (Gekle et al., 1994). Both rIMCD and MDCK cells were cultured in DMEM supplemented with 29 mM sodium bicarbonate, 8 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco Laboratories, Burlington, Ont., Canada). The cells were passaged upon reaching subconfluent density by treatment in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline with 0.1% trypsin from Sigma (C7- and C11-MDCK) or with 0.05% trypsin from Gibco Laboratories (all other types of REC) and scraped from the flasks with a rubber policeman. Dispersed cells were counted and inoculated at 1.25×10^3 cell/cm^2. Both stock cultures and cultures for experiments were grown for 6–8 days to attain subconfluency, in 80 cm^2 culture flasks and 6- or 24-well plates, respectively. In part of the experiments (Figs. 8 and 9), cells were serum-starved for 2 days in DMEM containing 0.1% BSA.

Table 1. Baseline activity of Na^+,K^+ pump, Na^+,K^+,Cl^- cotransport and (ouabain + bumetanide)-resistant ^{86}Rb influx in epithelial cells from rabbit, rat and canine kidneys

Type of cells	*n*	Na^+,K^+ pump	Na^+,K^+,Cl^- cotransport	Ouabain + bumetanide-resistant ^{86}Rb influx
		nmol per mg of protein per 15 min		
Rabbit PT	8	51 ± 7	107 ± 15	33 ± 5
Rabbit DT	7	35 ± 21	76 ± 13	28 ± 4
Rabbit CD	6	17 ± 6	46 ± 3	9 ± 1
Rat IMCD	7	83 ± 6	50 ± 5	7 ± 1
MDCK	5	60 ± 4	28 ± 3	5 ± 1
C7-MDCK	6	16 ± 8	189 ± 17	7 ± 2
C11-MDCK	8	54 ± 3	16 ± 2	4 ± 1

Means ± SE obtained in *n* experiments performed in triplicate or quadruplicate are given.

Measurement of K^+ (^{86}Rb) Influx

Cells growing in 24-well plates were washed twice with 2 ml of medium A containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-tris buffer (pH 7.4, room temperature) and incubated for 30 min at 37°C in 1 ml of medium B with or without ATP and other compounds listed in the table and figure legends. Medium B contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5 and HEPES-tris 20 (pH 7.4). The preincubation medium was replaced by 0.25 ml of the same medium with or without 1 mM ouabain and 20 μM bumetanide. The cells were incubated at 37°C for 5 min, and 0.25 ml of medium B containing 1–2 μCi/ml $^{86}RbCl$ was added thereafter. ^{86}Rb uptake was terminated by the addition of 2 ml of ice-cold medium C containing 100 mM $MgCl_2$ and 10 mM HEPES-tris buffer (pH 7.4). The cells were then transferred on ice, washed 4 times with 2 ml of ice-cold medium C and lysed with 1 ml of 1% SDS/4 mM EDTA mixture. The radioactivity of the cell lysate was measured with a liquid scintillation analyzer. ^{86}Rb (K^+) influx was calculated as $V = A/amt$ where A is the radioactivity in the sample (cpm), a is the specific radioactivity of ^{86}Rb (K^+) (cpm/nmol) in the incubation medium, m is the protein content in the sample (mg) and t is the incubation time (min). Protein content was measured by Lowry's method. As reported previously, the kinetics of ^{86}Rb uptake by MDCK cells were linear up to at least 20 min (Gagnon et al., 1998). Unless otherwise indicated, an incubation time of 15 min was used to determine the initial rate of K^+ influx. The activity of Na^+,K^+ pump and Na^+,K^+,Cl^- cotransport was estimated as the rate of ouabain-sensitive and ouabain-resistant, bumetanide-sensitive ^{86}Rb influx, respectively. Table 1 presents baseline values of the activity of Na^+,K^+ pump, Na^+,K^+,Cl^- cotransport and (ouabain + bumetanide)-resistant ^{86}Rb influx in REC used in this study.

We have reported previously that the addition of ouabain does not affect the absolute values of Na^+,K^+,Cl^- cotransport in MDCK cells (Gagnon et al., 1999*a*).

Intracellular Free Calcium Concentration

Cells growing in 80 cm^2 flasks were lifted by trypsin treatment, as described above, and washed twice in DMEM containing 10% calf serum, followed by 2 washes in medium B. Cells resuspended in 3 ml of medium B were incubated for 1 hr at 37°C in the presence of 5 μM fluo 3 AM and 0.02% pluronic F-127 with permanent stirring. They were centrifuged (800 × *g*, 3 min), washed twice with medium B containing 1% BSA and 2.5 mM probenecid, then kept in 3 ml of the same medium at room temperature for no more than 3 hr. Before the measurement of fluorescence (*F*), 1 ml of cell suspension was centrifuged and the cells were washed with medium B containing 1 mM probenecid, then resuspended in 2.5 ml of the same medium. *F* was measured at λ_{ex} = 483 nm and λ_{em} = 523 nm (slits 1 and 9 nm, respectively), using a SPEX FluoroMax spectrophotofluorometer (Edison, NJ). Free intracellular Ca^{2+} concentration was quantified as $[Ca^{2+}]_i = K_d (F - F_{min}) (F_{max} - F)^{-1}$, where F_{max} and F_{min} are maximal and minimal values of *F* measured in the presence of 0.5% triton X-100 and 2 mM $CaCl_2$ or 10 mM EGTA (pH 8.9), respectively; and K_d is the dissociation constant of the Ca^{2+}-fluo 3 complex (864 nM at 37°C (Merritt et al., 1990)).

Inositol Triphosphate Production

Cells seeded on 24-well plates were prelabeled overnight with 3 μCi/ml *myo*-[2-^{3}H]-inositol. Prior to the experiment, the radioactive medium was aspirated, and the cells were washed 3 times with 1 ml of medium A, followed by 30-min preincubation at 37°C in medium D containing 130 mM NaCl, 15 mM LiCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 20 mM HEPES-tris (pH 7.4). This medium was replaced thereafter by 0.25 ml of medium D for 30 min at 37°C, followed by the addition of 0.25 ml of medium B containing 200 μM ATP for 5 min. Incubation was terminated by SDS/EDTA mixture. The cell lysates were applied to a column containing 0.5 g DOWEX-AG 1-X8 (formate form), and IP_3 was resolved as described elsewhere (Orlov et al., 1992).

cAMP Production

Cells seeded in 12-well plates were washed twice with medium A and incubated for 1 hr in 1 ml of medium B with or without 100 μM ATP, 10 μM forskolin and 10 μM indomethacin. This medium was aspirated and the cells were treated with 1 ml of 1N perchloric acid. cAMP production was determined as increment of cAMP content in the incubation medium and in cell lysates measured by radioimmunoassay (Hamet, Pang & Tremblay, 1989).

MAP Kinase Phosphorylation

Serum-starved cells grown in 6-well plates were stimulated with agonists for 10 min, washed twice with ice-cold phosphate-buffered saline and lysed in 150 μl of buffer containing 25 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 200 μM Na-orthovanadate and 1 mM NaF. The lysed cells were scraped and centrifuged at 14,000 rpm for 20 min in a microcentrifuge. An equal volume of clear lysates containing 20 μg of protein was applied on 10% polyacrylamide gel, followed by electrophoresis and transfer to Immobilon-P membrane (Millipore, Bedford, MA). Phosphorylation of MAP kinases was determined by western blot analysis with antibodies against phospho-ERK, phospho-JNK and phospho-p38, according to the manufacturers' instructions. The equal content of MAP kinase kinase substrates was confirmed by immunoblotting with anti-ERK, anti-JNK and anti-p38 antibodies.

Chemicals

PMA, ATP, ouabain, bumetanide, amiloride, probenecid, indomethacin, DOWEX-AG 1-X8, anti-cyclic AMP-2′-BSA antibodies, Sigma (St. Louis, MO); thapsigargin, U-73122, Calbiochem (La Jolla, CA); fluo 3AM, pluronic F-127, Molecular Probes (Eugene, OR); anti-phospho-ERK1/2 and anti-phospho-p38 antibodies, New England Biolab (Beverly, MA); anti-phospho-JNK, anti-JNK and anti-p38 antibodies, Santa Cruz Biotechnology, Santa Cruz, CA; ^{86}RbCl, *myo*-[2-^{3}H]-inositol, Dupont (Boston, MA); salts, D-glucose and buffers, Sigma and Anachemia (Montreal, Canada). Anti-p42 ERK antibodies were kindly provided by Dr. Michael J. Dunn (Medical College of Wisconsin, Milwaukee, WI).

Abbreviations

CD—collecting duct; DT—distal tubule; IP_3—inositol 1,4,5-triphosphate; MAPK—mitogen-activated protein kinases; MDCK—Madin-Darby canine kidney cells (wild type); C7- and C11-MDCK—subclones of MDCK cells possessing properties of principal and intercalated cells from CD; PKA and PKC—protein kinase A and C, respectively; PLA_2 and PLC—phospholipase A_2 and C; PMA—4β-phorbol 12-myristate 13-acetate; PT—proximal tubule; REC—renal epithelial cells; rIMCD—rat inner medullary CD.

Results

Regulation of ^{86}Rb Influx by P_2 Purinoceptors in REC Derived from PT, DT and CD

We have reported previously that the addition of PMA and ATP slightly potentiated the activity of Na^+,K^+ pump in MDCK cells, whereas Na^+,K^+,Cl^- cotransport was completely inhibited by PMA, partially inhibited (~40–50%) by ATP and augmented by 30% under activation of adenylate cyclase with forskolin (Gagnon et al., 1998). Data obtained on MDCK cells in the present study confirmed these findings (Tables 2 and 3). In rabbit PT, Na^+,K^+ pump was inhibited by 35% with PMA and was activated by ~2-fold with forskolin. In contrast to PT, PMA augmented Na^+,K^+ pump by 2-fold in cells from rabbit DT. We did not observe significant effects of these compounds on Na^+,K^+ pump activity in rabbit CD cells and in rIMCD (Table 2). Activators of PKC and PKA have been shown to decrease the Na^+,K^+-ATPase in rat PT, measured as the rate of ATP hydrolysis in tissue homogenates, but do not affect the activity of this enzyme in CD and the thick ascending limb of

Table 2. Effect of PMA, ATP and forskolin on the activity of Na^+,K^+ pump in renal epithelial cells

Additions, μM	Na^+,K^+ pump, %				
	MDCK	Rabbit PT	Rabbit DT	Rabbit CD	rIMCD
None (control)	100 ± 8	100 ± 6	100 ± 7	100 ± 12	100 ± 5
PMA, 0.1	128 ± 11	65 ± 9[a]	203 ± 16[c]	108 ± 14	116 ± 4
ATP, 100	132 ± 8[a]	102 ± 15	142 ± 45	113 ± 8	123 ± 8
Forskolin, 10	107 ± 10	182 ± 33[b]	120 ± 17	98 ± 16	106 ± 5

Cells were preincubated for 30 min with compounds listed in the left column. Thereafter, the medium was aspirated and 0.25 ml of the same medium with or without 1 mM ouabain was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1–2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+ pump in the absence of any additions was taken as 100%. Means ± SE obtained in 3 experiments performed in triplicate or quadruplicate are given. [a], [b], [c] -$P < 0.05$, 0.02 and 0.01, respectively, compared with control values.

Henle's loop (Satoh, Cohen & Katz, 1993; Ominato, Satoh & Katz, 1996). In contrast, in both intact opossum kidney cells derived from PT (Pedemonte et al., 1997*a*) and in the same cells transfected with the α1-isoform of Na^+,K^+-ATPase (Pedemonte et al., 1997*b*), PMA transiently activates this ion transport pathway, measured as the rate of ouabain-sensitive ^{86}Rb uptake. Possible explanations for the controversial data obtained in these studies have been considered recently (Gagnon et al., 1999*b*).

As in MDCK cells, PMA caused virtually complete supression of Na^+,K^+,Cl^- cotransport in rIMCD cells derived from rat CD and inhibited this carrier by 30–50% in REC from different segments of the rabbit tubule (Table 3). In contrast to PMA, ATP did not affect Na^+,K^+,Cl^- cotransport in REC from PT and DT but decreased it by ~50% in MDCK cells and by ~30% in cells from rabbit and rat CD (Table 3). In these cells, the level of ATP-induced inhibition of Na^+,K^+,Cl^- cotransport was not affected by the presence of ouabain (*data not shown*).

Regulation of $[Ca^{2+}]_i$ by P_2 Purinoceptors in REC Derived from PT, DT and CD

The lack of effect of ATP on Na^+,K^+,Cl^- cotransport in REC derived from PT and DT may be caused by the absence of functional P_2 receptors. It has been shown that activation of all types of P_{2Y} receptors cloned so far is accompanied by triggering of phospholipase C (PLC) and Ca^{2+}_i release (Burnstock & King, 1996; Brake & Julius, 1996). Considering this, we compared the effect of ATP on $[Ca^{2+}]_i$ in REC derived from different segments of the rabbit tubule. The addition of ATP led to a transient elevation of $[Ca^{2+}]_i$ in REC from CD showing inhibition of Na^+,K^+,Cl^- cotransporter by ATP as well as in REC from PT and DT lacking an ATP-sensitive carrier (Fig. 1, Table 3). The higher baseline $[Ca^{2+}]_i$ values in fluo 3-loaded REC (~400 nM, Fig. 1) compared to fura 2-loaded cells (100–200 nM (Burnstock & King, 1996; Brake & Julius, 1996)) are in accordance with data obtained for other cell types (Merritt et al., 1990) and are caused by different K_d values of fluo 3-Ca and fura- 2-Ca complexes (864 and 224 nM, respectively).

Regulation of ^{86}Rb Influx by P_2 Purinoceptors in C7- and C11-MDCK Cells

The CD of mammalian kidneys is a heterogeneous epithelium consisting of principal and intercalated cells with different morphological and functional properties. Principal cells are mainly involved in K^+ secretion and Na^+ reabsorption, whereas intercalated cells are responsible for acid-base transepithelial transport (Hamm & Alpern, 1992; Koeppen & Stanton, 1992; Schwartz, 1995). Interestingly, two substrains of cells with different morphological and electrical properties have been identified in commercially available stocks of MDCK (Richardson, Scalera & Simmons, 1981; Barker & Simmons, 1981). These two subtypes of MDCK cells, with high (C7-MDCK) and low (C11-MDCK) transepithelial electrical resistance (R_{te} ~5,000–6,000 Ω/cm^2 and 300–400 Ω/cm^2) and possessing the functional properties of principal and intercalated cells, respectively, have been cloned from wildtype MDCK (Gekle et al., 1994). We used these substrains to study the relative contributions of principal and intercalated cells in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport revealed in REC derived from canine, rabbit and rat CD (Table 3).

In C7- as well as in C11-MDCK, PMA led to virtually complete suppression of Na^+,K^+,Cl^- cotransport (Fig. 2). In contrast, ATP decreased the activity of Na^+,K^+,Cl^- cotransport in C11-MDCK but did not significantly affect this carrier in C7-MDCK. In both subtypes of MDCK cells, Na^+,K^+,Cl^- cotransport was insensitive to forskolin. Figure 3 charts the time course of inhibition of Na^+,K^+,Cl^- cotransport in C11 cells by PMA and ATP. In both cases, maximal suppression was

Table 3. Effect of PMA, ATP and forskolin on the activity of Na^+,K^+,Cl^- cotransport in renal epithelial cells

Additions, μM	Na^+,K^+,Cl^- cotransport, %				
	MDCK	Rabbit PT	Rabbit DT	Rabbit CD	rIMCD
None (control)	100 ± 6	100 ± 8	100 ± 10	100 ± 7	100 ± 7
PMA, 0.1	7 ± 6[c]	68 ± 5[a]	55 ± 4[b]	69 ± 3[c]	9 ± 1[c]
ATP, 100	52 ± 8[b]	89 ± 6	94 ± 5	71 ± 8[a]	72 ± 8[a]
Forskolin, 10	129 ± 10	112 ± 24	128 ± 6	150 ± 11[a]	90 ± 6

Cells were preincubated for 30 min with compounds listed in the left column. Thereafter, the medium was aspirated and 0.25 ml of the same medium containing 1 mM ouabain with or without 20μM bumetanide was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1–2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+ pump in the absence of any additions was taken as 100%. Means ± SE obtained in 3 experiments performed in triplicate or quadruplicate are given. [a], [b], [c] $-P < 0.05$, 0.02 and 0.001, respectively, compared with control values.

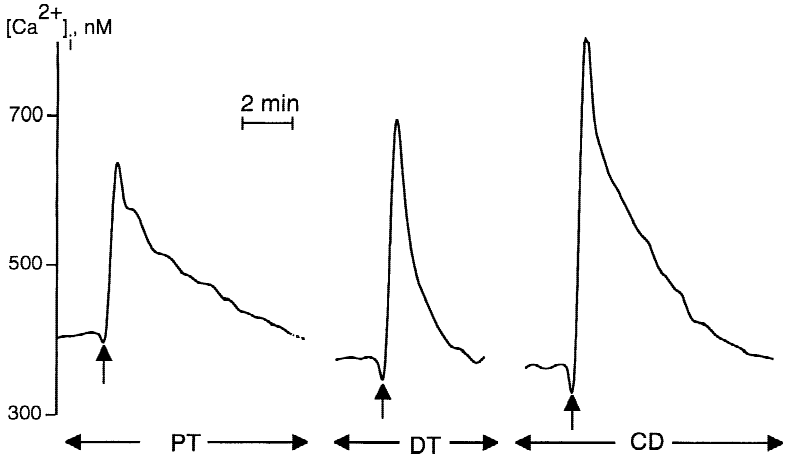

Fig. 1. Effect of ATP on intracellular free calcium concentration ($[Ca^{2+}]_i$) in fluo 3-loaded epithelial cells derived from proximal tubules (PT), distal tubules (DT) and collecting ducts (CD) from the rabbit kidney. Additions of ATP (100 μM) are shown by arrows.

observed after 30 min of preincubation of cells with these compounds. Based on these results, we limited the time of preincubation with ATP by 30 min in subsequent experiments.

As seen in Fig. 4, none of the test compounds affected Na^+,K^+-pump in C11-MDCK cells. In contrast, the activity of Na^+,K^+-pump in C7-MDCK cells was augmented by ~3-, 9- and 12-fold under the addition of PMA, ATP and forskolin, respectively. Using C7- and C11-MDCK, we did not observe the effect of these compounds on (ouabain + bumetanide)-resistant ^{86}Rb influx (*data not shown*).

Signaling Systems in C7- and C11-MDCK Cells Triggered by P_2 Receptors

It is well-documented that activation of P_2 purinoceptors in MDCK cells leads to augmentation of PLC activity and IP_3 accumulation (Balboa et al., 1994; Firestein et al., 1996; Insel et al., 1996; Gagnon et al., 1999*a*), a transient elevation of $[Ca^{2+}]_i$ (Paulmichl et al., 1991; Delles et al., 1995; Gagnon et al., 1999*a*), cAMP production by phospholipase A_2 (PLA_2)-dependent and -independent pathways (Paulmichl et al., 1991; Firestein et al., 1996; Insel et al., 1996) and phosphorylation of ERK1/2 (p42/p44) MAP kinases (Xing et al., 1997; Gagnon et al., 1999*a*). To examine the possible contributions of these signaling pathways in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport, we compared their efficiency in C7-MDCK and C11-MDCK cells.

Figure 5 shows that ATP-induced IP_3 production was ~2-fold higher in C11 compared with C7 cells; this difference was completely abolished by the PLC inhibitor U73122. Forskolin-induced cAMP production in C7 and C11 cells was not different and was not affected by an inhibitor of cyclooxygenase, indomethacin (Fig. 6). In the absence of indomethacin, ATP-induced cAMP production was similar in C7 and C11 cells. Indomethacin decreased cAMP production in C7 and C11 cells by 25 and 50%, respectively, thus showing the higher contribution of the PLA_2/cyclooxygenase-mediated pathway in the ATP-induced cAMP response in intercalated cells.

We did not observe any significant differences in maximal $[Ca^{2+}]_i$ elevation (Fig. 7 *a* and *c*), but the du-

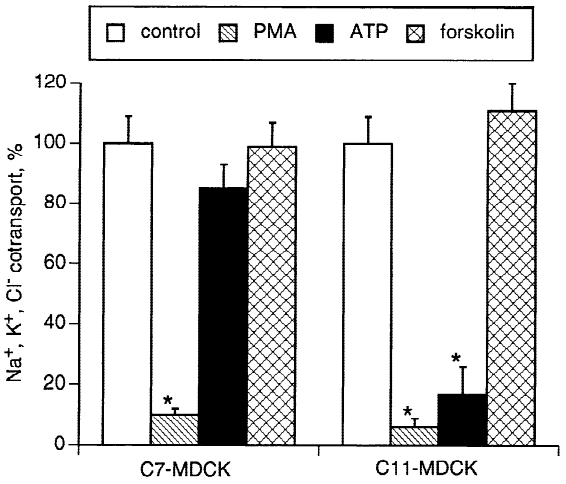

Fig. 2. Effect of PMA, ATP and forskolin on the activity of Na^+,K^+,Cl^- cotransport in C7- and C11-MDCK. The cells were preincubated with 0.1 μM PMA, 100 μM ATP or 10 μM forskolin for 30 min. Thereafter, the medium was aspirated, and 0.25 ml of the same medium containing 1 mM ouabain with or without 20 μM bumetanide was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1–2 μCi/ml $^{86}RbCl$ and terminated in 15 min. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± SE obtained in 4 experiments performed in triplicate or quadruplicate are given. *—$P < 0.001$ compared with control values.

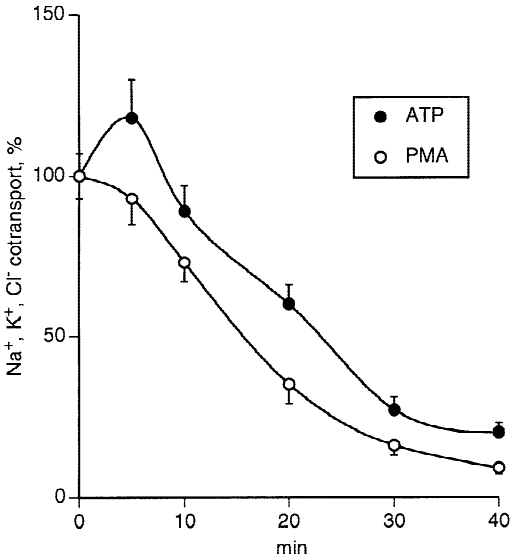

Fig. 3. Kinetics of the inhibition of Na^+,K^+,Cl^- cotransport in C11-MDCK cells by ATP and PMA. The cells were preincubated with 0.5 ml of medium B up to 1 hr and in the last 40, 30, 20, 10 and 5 min of preincubation, 50 μl of 1 μM PMA or 1 mM ATP was added. Then, ^{86}Rb uptake was initiated by the addition of 0.5 ml of medium B with 4 μCi/ml $^{86}RbCl$, 1 mM ouabain ± 20 μM bumetanide and terminated in 5 min. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± SE obtained in experiments performed in quadruplicate are given.

ration of the ATP-induced $Ca^{2+}{}_i$ signal was markedly increased in C11-MDCK compared to C7-MDCK cells. This difference may be caused by an enhanced rate of Ca^{2+} extrusion from C7 cells by plasma membrane Ca^{2+}-pump and/or Ca^{2+}/Na^+ exchanger or by quenched activity in these cells of calcium release-activated channels (CRAC) which are widely expressed in various types of non-excitable cells (Holda et al., 1998). The latter hypothesis is supported by the following data. As seen in Fig. 7*b* and *d*, the difference in kinetics of the ATP-induced $[Ca^{2+}]_i$ elevation between C7 and C11 cells was abolished in Ca^{2+}-free medium; the addition of $[Ca^{2+}]_o$ led to a sustained rise of $[Ca^{2+}]_i$ in ATP-pretreated C11-MDCK and only slightly augmented this parameter in C7-MDCK cells.

The most dramatic differences between C7 and C11 cells were observed in the study of MAP kinase phosphorylation. In C7-MDCK, ERK phosphorylation was increased sharply by PMA but was not affected by ATP (Fig. 8*a*). In contrast, in C11-MDCK, ERK phosphorylation was potentiated sharply by ATP and to a much lesser extent by PMA. The level of ERK phosphorylation by ATP and PMA in rIMCD was similar. In all types of REC, an elevation of $[Ca^{2+}]_i$ by thapsigargin did not significantly affect ERK phosphorylation. We did not detect any effect of ATP, PMA and thapsigargin on JNK1 phosphorylation in C7-MDCK (Fig. 9*Aa*). In C11-MDCK, on the other hand, JNK1 phosphorylation was augmented sharply by ATP and moderately by PMA and thapsigargin. Modest activation of JNK1 phosphorylation by ATP and thapsigargin was seen in rIMCD. In contrast to ERK1/2 and JNK1, these compounds did not alter p38 phosphorylation (Fig. 9*Ba*).

Discussion

The data obtained in the present study show that the recently discovered inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells by agonists of P_{2Y} receptors (Gagnon et al., 1998) is a feature of REC from CD. As with our findings in CD-derived MDCK cells, we noted that ATP inhibited Na^+,K^+,Cl^- cotransport in REC from rabbit and rat CD but did not affect this carrier in cells from PT and DT (Table 3). It should be underlined that MDCK cells with high transepithelial resistance (3,000–6,000 Ω/cm^2) were used in an overwhelming majority of studies on intracellular signaling triggered by P_2 receptors. In contrast, the MDCK strain from ATTC employed in our previous experiments displayed low transepithelial resistance (197 ± 47 Ω/cm^2) (Gagnon et al., 1998). We, therefore, suggested that P_{2Y} receptor-induced inhibition of Na^+,K^+,Cl^- cotransport seen in our study was caused by an abundance of intercalated cells in our MDCK stock (Gagnon et al., 1999*a*). The results obtained with C7- and C11-MDCK cells confirmed this hypothesis.

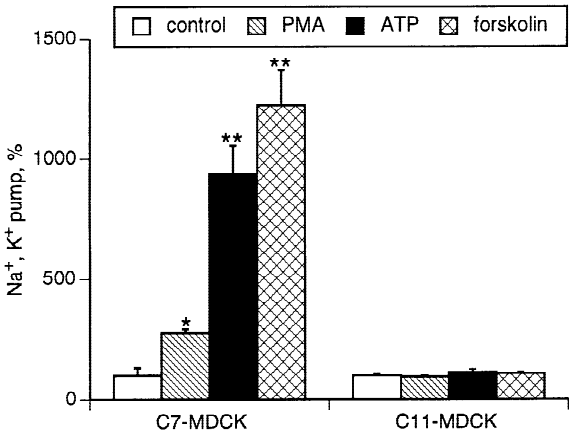

Fig. 4. Effect of PMA, ATP and forskolin on the activity of Na^+,K^+ pump in C7- and C11-MDCK. The cells were preincubated with 0.1 μM PMA, 100 μM ATP or 10 μM forskolin for 30 min. Thereafter, the medium was aspirated, and 0.25 ml of the same medium with or without 1 mM ouabain was added. After 5 min, ^{86}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1–2 μCi/ml $^{86}RbCl$ and terminated in 15 min. The value of Na^+,K^+ pump in the absence of any additions was taken as 100%. Means ± SE obtained in 4 experiments performed in triplicate or quadruplicate are given. *, **-$P < 0.005$ and 0.001 compared with control values, respectively.

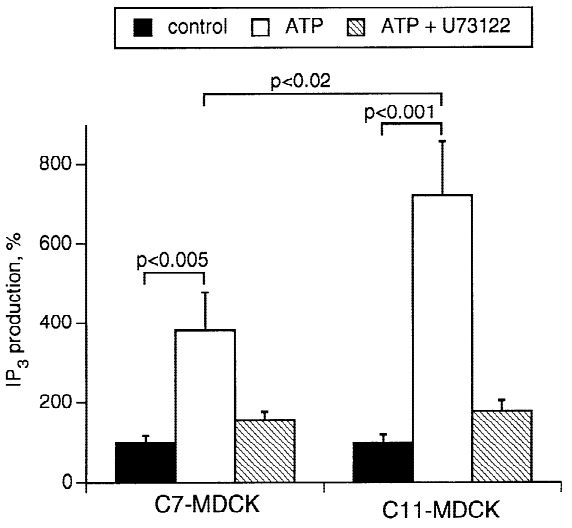

Fig. 5. Effect of ATP and U73122 on inositol-1,4,5-triphosphate (IP_3) production in C7- and C11-MDCK cells. The cells were preincubated with or without 10 μM of U73122 IP_3 for 30 min, followed by stimulation with 100 μM ATP for 5 min. IP3 production in ATP- and U73122-untreated cells was taken as 100%. Means ± SE obtained in experiments performed in quadruplicate are given.

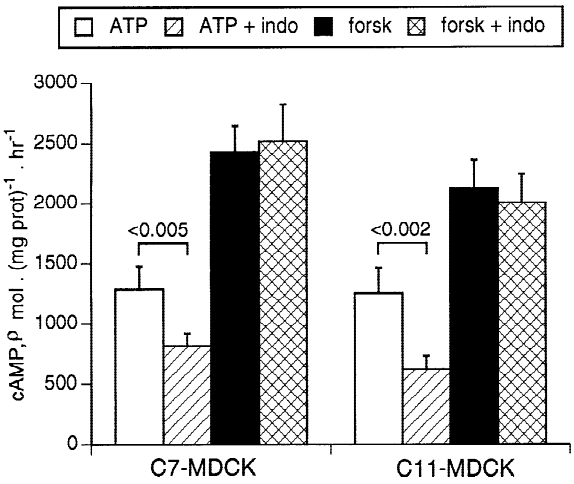

Fig. 6. Effect of ATP (100 μM), indomethacin (indo—10 μM) and forskolin (forsk—10 μM) on cAMP production in C7- and C11-MDCK cells. The cells were incubated with the above-listed compounds for 1 hr, and cAMP content was measured as described in Materials and Methods. Baseline cAMP production in C7 and C11 cells was not significantly different (5.3 ± 1.7 and 6.3 ± 2.0 pmol per mg of protein per hr, respectively). Means ± SE obtained in experiments performed in quadruplicate are given.

It has been shown previously that C11-MDCK cells with low transepithelial resistance are peanut-lectin (PNA)-positive, maintain pH_i at 7.16 and secrete Cl^- and H^+ in apical media. In contrast, C7 cells with high transepithelial resistance are PNA-negative, maintain pH_i at 7.39 and secrete K^+ (Gekle et al., 1994). These results reveal that C7-MDCK and C11-MDCK are highly abundant with principal and intercalated cells, respectively. ATP decreased Na^+,K^+,Cl^- cotransport by 80–90% in C11-MDCK but did not alter its activity in C7-MDCK (Fig. 2). These results strongly indicate that inhibition of Na^+,K^+,Cl^- cotransport by P_2 receptors is limited to intercalated cells from CD.

It might be suggested that the lack of effect of ATP on Na^+,K^+,Cl^- cotransport in C7-MDCK, resembling principal cells from CD, as well as in REC from PT and DT is caused by the absence of functional P_{2Y} receptors. However, our data contradict this. MDCK cells (Gagnon et al., 1999*a*), C7-MDCK (Fig. 7) and REC derived from rabbit PT and DT (Fig. 1) displayed an ATP-induced transient increment of $[Ca^{2+}]_i$. Similar results were obtained in a study on the effect of ATP on $[Ca^{2+}]_i$ in REC from rabbit PT (Romero et al., 1992) as well as in freshly isolated rat PT (Cha, Sekine & Endou, 1998) and CD (Ecelbarger et al., 1994; Cha et al., 1998). In addition to $Ca^{2+}{}_i$-signaling in C7 cells, ATP caused an elevation of IP_3 (Fig. 5) and cAMP (Fig. 6) production and sharply augmented Na^+,K^+ pump activity (Fig. 4). These data indicate that P_{2Y} receptors coupled to PLC-mediated Ca^{2+}-signaling and cAMP elevation are expressed in all segments of the mammalian nephron, whereas P_{2Y} receptor-sensitive Na^+,K^+,Cl^- cotransport is limited to intercalated-like C11-MDCK cells.

It may be assumed that purinergic inhibition of Na^+,K^+,Cl^- cotransport observed in C11-MDCK cells is caused (i) by the presence of special isoforms of the carrier or P_{2Y} receptors which are selectively expressed

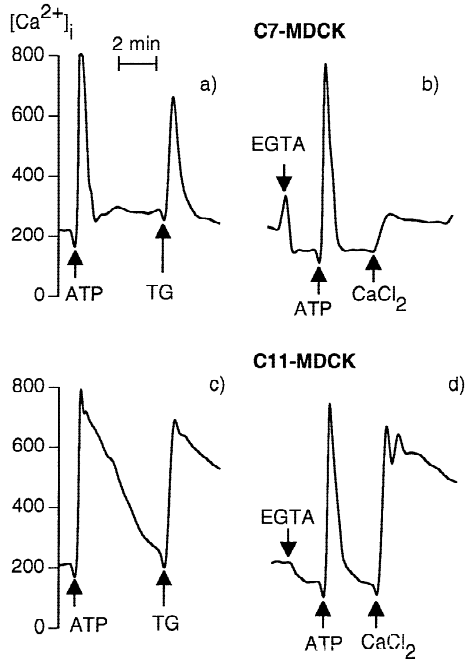

Fig. 7. Effect of ATP and thapsigargin on intracellular free calcium concentration ($[Ca^{2+}]_i$) in C7-MDCK cells (*a* and *b*) and C11-MDCK cells (*c* and *d*). Fluo 3-loaded cells were incubated in medium B, and 100 μM ATP, 0.5 μM thapsigargin (TG), 1.2 mM EGTA and 3 mM $CaCl_2$ were added at the times indicated by arrows.

in these cells; or (ii) by peculiarities of P_{2Y} receptor-triggered signaling pathways. Two isoforms of Na^+,K^+,Cl^- cotransport have been cloned up to now. The renal-specific isoform of Na^+,K^+,Cl^- cotransporter (NKCC2) is selectively expressed in apical membranes of the thick ascending limb of Henle's loop and DT, whereas NKCC1 is expressed in all types of nonepithelial cells studied so far, including basolateral membranes of REC (Payne & Forbush, 1994; Payne et al., 1995; Kaplan et al., 1996*b*), CD (Kaplan et al., 1996*a*) and particularly MDCK cells (Deppe et al., 1997). The targeted expression of NKCC1 in REC from CD is also supported by the basolateral location of bumetanide-sensitive ion fluxes in MDCK monolayers (Aiton et al., 1982; Simmons, Brown & Rug, 1984). Using monoclonal antibodies against a 74-amino acid carboxy-terminal tail of NKCC1, Kaplan and coworkers (1996*a*) demonstrated that this isoform of Na^+,K^+,Cl^- cotransporter is not uniformly expressed along the nephron, and the CD of the mouse kidney is highly abundant with this carrier compared to other tubular segments. In contrast to this observation, we showed that the absolute values of bumetanide-sensitive ^{86}Rb influx are higher in REC from rabbit PT vs. DT and CD (Table 1). To explain this contradiction we propose that (i) the sequence of C-terminus used as epitope for NKCC1 antibodies is different in PT and CD because of post-translation modification or alternative splicing; (ii) the pattern of NKCC1 expression along the tubule is species-dependent; and (iii) the level of NKCC1 expression is potentiated in cultured REC from PT. The lack of data on the sequence of P_{2Y} receptors from the canine cDNA library does not allow comparison of the expression of this superfamily in C7- and C11-MDCK.

As mentioned in the Results, stimulation of P_{2Y} receptors leads to PLC activation coupled to IP_3 and diacylglycerol production. IP_3 production causes intracellular Ca^{2+} release, whereas diacylglycerol activates PKC. In MDCK cells, this signaling pathway is also coupled to activation of PLA_2 and PGE_2 release. Using a pharmacological approach, Post and coworkers (1998) demonstrated that in MDCK cells with high transepithelial resistance, PLC-mediated signaling is triggered by P_{2Y2} receptors, whereas P_{2Y1} and P_{2Y11} receptors contribute to PLA_2-independent cAMP production. Employing modulators of Ca^{2+}-signaling (BAPTA, thapsigargin, A23187), PLC (U73122) and PLA_2 (arachidonic acid, indomethacin, NDGA, $AACOCF_3$) as well as an activator of cAMP signaling (forskolin) and inhibitors of PKC (staurosporin, calphostin C, chronic PMA treatment), we showed that none of the above-listed signaling pathways is involved in the supression of Na^+,K^+,Cl^- cotransport in MDCK cells by agonists of P_2 purinoceptors (Gagnon et al., 1998; 1999*a*). The present study demonstrated about the same effect of ATP on $[Ca^{2+}]_i$ elevation, cAMP and IP_3 production (Figs. 5–7) in C11- and C7-MDCK cells, possessing and lacking ATP-sensitive Na^+,K^+,Cl^- cotransport, respectively, further confirming this conclusion.

In the last few years, it has been documented that besides receptor tyrosine kinases, G protein-coupled receptors are able to activate the MAP kinase cascade (Rocca et al., 1997). It has been demonstrated recently that activation of P_{2Y} receptors by ATP leads to ERK1/2 phosphorylation in MDCK cells (Xing et al., 1997; Gagnon et al., 1999*a*). In the present study, we showed that apart from ERK (Fig. 8), extracellular ATP is also able to induce JNK1 phosphorylation (Fig. 9*A*) but does not affect phosphorylation of p38, another member of stress-activated MAP kinases (Fig. 9*B*). ATP-induced phosphorylation of ERK and JNK has been observed in C11-MDCK, and rIMCD where ATP inhibits Na^+,K^+,Cl^- cotransporter. In contrast, in C7 cells lacking ATP-sensitive Na^+,K^+,Cl^- cotransporter, ATP does not affect phosphorylation of these MAP kinases. Our results suggest that purinergic regulation of Na^+,K^+,Cl^- cotransporter is mediated by activation of the MAP kinase signaling cascade.

Transcriptional factors are the best studied substrates of MAP kinases. It is well-documented that ERK phosphorylates ternary complex factor/Elk-1, which regulates c-*fos* induction, whereas JNK phosphorylates

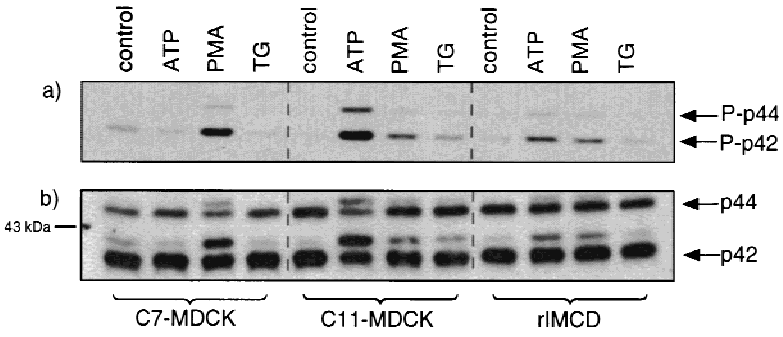

Fig. 8. Effect of ATP, PMA and thapsigargin on phosphorylation of MAP kinase ERK1 (p42)/ERK2 (p44) in C7-MDCK, C11-MDCK and rIMCD cells. Phosphorylation of MAPK was assessed by gel retardation of phosphorylated ERK1/2 using anti-p42/p44 antibodies (*b*), or by immunoblotting with phospho-specific anti-42/p44 antibodies (*a*). The cells were incubated with 100 μM ATP, 0.1 μM PMA or 0.5 μM thapsigargin for 10 min. For more details *see* Materials and Methods.

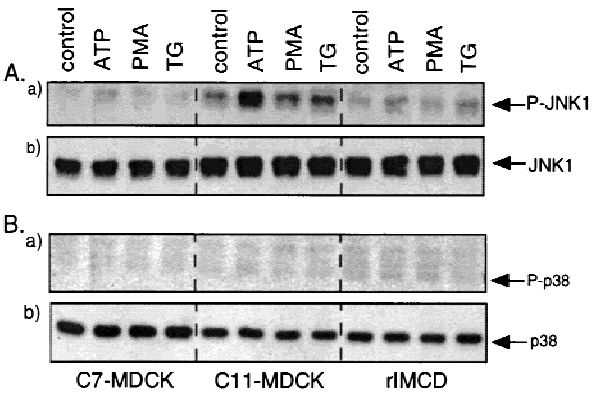

Fig. 9. Effect of ATP, PMA and thapsigargin on phosphorylation of stress-activated protein kinase JNK1 (*A*) and p38 MAP kinase (*B*) in C7-MDCK, C11-MDCK and rIMCD cells. Phosphorylation of JNK and p38 was assessed by immunoblotting with phospho-specific anti-JNK1 and anti-p38 antibodies, respectively (*a*). The equal amount of JNK1 and p38 in cell lysates was confirmed by immunoblotting with antibodies against JNK and p38, respectively (*b*). Cells were incubated with 100 μM ATP, 0.1 μM PMA or 0.5 μM thapsigargin for 10 min. For more details *see* Materials and Methods.

N-terminal c-Jun and activating transcriptional factor 2 (Vojtek & Der, 1998). Activation of the ERK-triggered pathway can be blocked selectively by, PD98059, an inhibitor of ERK kinase MEK; specific inhibitors of JNK kinase are currently not available. Using MDCK, we demonstrated that 50 μM PD98059 inhibited ATP-induced ERK1/2 phosphorylation but did not affect regulation of Na^+,K^+,Cl^- cotransport (Gagnon et al., 1999*a*). We, therefore, hypothesize that purinergic inhibition of Na^+,K^+,Cl^- cotransport in intercalated cells may be mediated by the MAP kinase cascade at a step upstream of MEK. This hypothesis is currently being examined in our laboratory.

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References

Aiton, J.F., Brown, C.D.A., Ogden, P., Simmons, N.L. 1982. K^+ transport in "tight" epithelial monolayers of MDCK cells. *J. Membrane Biol.* **65:**99–109

Balboa, M.A., Firestein, B.L., Godson, C., Bell, K.S., Insel, P.A. 1994. Protein kinase C-α mediates phospholipase D activation by nucleotides and phorbol ester in Madin-Darby canine kidney cells. *J. Biol. Chem.* **269:**10551–10561

Barker, G., Simmons, N.L. 1981. Identification of two strains of cultured canine renal epithelial cells (MDCK cells) which display entirely different physiological properties. *Q. J. Exp. Physiol.* **66:**61–72

Bohmann, C., Rump, L.C., Schaible, U., von Kugelgen, I. 1995. α-Adrenoceptor modulation of norepinephrine and ATP release in isolated kidneys of spontaneously hypertensive rats. *Hypertension* **25:**1224–1231

Brake, A.J., Julius, D. 1996. Signaling by extracellular nucleotides. *Annu. Rev. Cell. Dev. Biol.* **12:**519–541

Burnstock, G., King, B.F. 1996. Numbering of cloned P_2 purinoceptors. *Drug Develop. Res.* **38:**67–71

Cha, S.H., Sekine, T., Endou, H. 1998. P_2 purinoceptor localization along rat nephron and evidence suggesting existence of subtypes P2Y1 and P2Y2. *Am. J. Physiol.* **274:**F1006–F1014

Chan, C.M., Unwin, R.J., Bardini, M., Oglesby, I.B., Ford, A.P., Townsend-Nicholson, A., Burnstock, G. 1998. Localization of P2X1 purinoceptors by autoradiography and immunohistochemistry in rat kidneys. *Am. J. Physiol.* **274:**F799–F804

Communi, D., Govaerts, C., Parmentier, M., Boyenaems, J.-M. 1997. Cloning of human purinergic P_{2Y} receptors coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.* **272:**31969–31973

Delles, C., Haller, T., Dietl, P. 1995. A highly calcium-selective cation current activated by intracellular calcium release in MDCK cells. *J. Physiol.* **486:**557–569

Deppe, C.E., Heering, P.J., Tinel, H., Kinne-Saffran, E., Grabensee, B., Kinne, R.K.H. 1997. Effect of cyclosporine A on Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and H^+/K^+-ATPase in MDCK cells and two subtypes, C7 and C11. *Exp. Nephrol.* **5:**471–480

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

Firestein, B.L., Xing, M., Hughes, R.J., Corvera, C.U., Insel, P.A. 1996. Heterogeneity of P_{2U}- and P_{2Y}-purinergic receptor regulation of phospholipases in MDCK cells. *Am. J. Physiol.* **271:**F610–F618

Friedrich, F., Weiss, H., Paulmichl, M., Lang, F. 1989. Activation of potassium channels in renal epithelioid cells (MDCK) by external ATP. *Am. J. Physiol.* **256:**C1016–C1021

Gagnon, F., Dulin, N.O., Tremblay, J., Hamet, P., Orlov, S.N. 1999*a*.

ATP-induced inhibition of Na^+,K^+,Cl^- cotransport in Madin-Darby canine kidney cells: lack of involvement of known purinoceptor-coupled signaling pathways. *J. Membrane Biol.* **167:**193–204

Gagnon, F., Hamet, P., Orlov, S.N. 1999*b*. Na^+,K^+ pump and Na^+-coupled ion carriers in isolated mammalian kidney epithelial cells: regulation by protein kinase C. *Can. J. Physiol. Pharmacol.* **77:**305–319

Gagnon, F., Orlov, S.N., Tremblay, J., Hamet, P. 1998. Complete inhibition of Na^+,K^+,Cl^- cotransport in Madin-Darby canine kidney cells by PMA-sensitive protein kinase C. *Biochim. Biophys. Acta* **1369:**233–239

Gekle, M., Wunsch, S., Obelrleithner, H., Silbernagl, S. 1994. Characterization of two MDCK-cell subtypes as a model system to study principal cell and intercalated cell properties. *Pfluegers Arch.* **428:**157–162

Hamet, P., Pang, S.C., Tremblay, J. 1989. Atrial natriuretic factor-induced egression of cyclic guanosine 3′,5′-monophosphate in cultured vascular smooth muscle cells. *J. Biol. Chem.* **264:**12364–12369

Hamm, L.I., Alpern, R.J. 1992. Cellular mechanism of renal tubular acidification. *In:* The Kidney: Physiology and Pathophysiology. D.W. Seldin and G. Giebisch, editors. Raven Press, New York pp. 2581–2626

Holda, J.R., Klishin, A., Sedova, M., Huser, J., Blatter, L.A. 1998. Capacitative calcium entry. *News Physiol. Sci.* **13:**157–163

Insel, P.A., Firestein, B.L., Xing, M., Post, S.R., Jacobson, J.P., Balboa, M.A., Hughes, R.J. 1996. P_2-purinoceptors utilize multiple signalling pathways in MDCK-D1 cells. *J. Autonom. Pharmacol.* **16:**311–313

Kaplan, M.R., Plotkin, M.D., Brown, D., Hebert, S.C., Delpire, E. 1996*a*. Expression of the mouse Na-K-2Cl cotransporter, mBSC2, in the terminal inner medullary collecting duct, the glomerular and extraglomerular mesangium, and glomerular afferent arteriole. *J. Clin. Invest.* **98:**723–730

Kaplan, M.R., Plotkin, M.D., Lee, W.S., Xu, Z., Lytton, J., Forbush, B.I. 1996*b*. Apical localization of the Na-K-2Cl cotransporter, rBSC1, on rat thick ascending limbs. *Kidney Int.* **49:**40–47

Koeppen, B.M., Stanton, B.A. 1992. Sodium chloride transport. Distal nephron. *In:* The Kidney: Physiology and Pathophysiology. D.W. Seldin, and G. Giebisch, editors. Raven Press, New York p. 2003–2039

Koop, D.R., Laethem, R.D., Goldner, A.L., Douglas, J.G. 1991. Cytochrome P450 expression and metabolism in isolated rabbit renal epithelium. *Methods in Enzymology* **206:**364–371

Merritt, J.E., McCarthy, S.A., Davies, M.P.A., Moores, K.E. 1990. Use of fluo-3 to measure cytosolic Ca^{2+} in platelets and neutrophils. Loading cells with dye, calibration of traces, masurements in the presence of plasma, and buffering of cytosolic Ca^{2+}. *Biochem. J.* **269:**513–519

Ominato, M., Satoh, T., Katz, A.I. 1996. Regulation of Na-K-ATPase activity in the proximal tubule: role of the protein kinase C pathway and eicosanoids. *J. Membrane Biol.* **152:**235–243

Orlov, S.N., Resink, T.J., Bernhardt, J., Buhler, F.R. 1992. Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells: dependence on medium osmolality and regulation by signalling systems. *J. Membrane Biol.* **126:**199–210

Paulmichl, M., Pfeilschifter, J., Woll, E., Lang, F. 1991. Cellular mechanisms of ATP-induced hyperpolarization in renal epitheloid MDCK cells. *J. Cell Physiol.* **147:**68–75

Payne, J.A., Forbush III, B. 1994. Alternatively spliced isoforms of the putative renal Na-K-Cl cotransporter are differently distributed within the rabbit kidney. *Proc. Natl. Acad. Sci. USA* **91:**4544–4548

Payne, J.A., Xu, J.C., Haas, M., Lytle, C.Y., Ward, D., Forbush III, B. 1995. Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na-K-Cl cotransporter in human colon. *J. Biol. Chem.* **270:**17977–17985

Pedemonte, C.H., Pressley, T.A., Cinelli, A.R., Lokhandwala, M.F. 1997*a*. Stimulation of protein kinase C rapidly reduces intracellular Na^+ concentration via the Na^+ pump in OK cells. *Mol. Pharmacol.* **52:**88–97

Pedemonte, C.H., Pressley, T.A., Lokhandwala, M.F., Cinelli, A.R. 1997*b*. Regulation of Na,K-ATPase transport activity by protein kinase C. *J. Membrane Biol.* **155:**219–227

Post, S.R., Rump, C., Zambon, A., Hughes, R.J., Buda, M.D., Jacobson, J.P., Kao, C.C., Insel, P.A. 1998. ATP activates cAMP production via multiple purinergic receptors in MDCK-D1 epithelial cells. *J. Biol. Chem.* **273:**23093–23097

Rice, W.R., Burton, F.M., Fiedeldey, D.T. 1995. Cloning and expression of the alveolar type II cell P_{2U}-purinergic receptor. *Am. J. Respir. Cell Mol. Biol.* **12:**27–32

Richardson, J.C.W., Scalera, V., Simmons, N.L. 1981. Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim. Biophys. Acta* **673:**26–36

Rocca, G.J.D., van Biesen, T., Daaka, Y., Luttrell, D.K., Luttrell, L.M., Lefkowitz, R.J. 1997. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. *J. Biol. Chem.* **272:**19125–19132

Romero, M.F., Douglas, J.G., Eckert, R.L., Hopfer, U., Jacobberger, J.W. 1992. Development and characterization of rabbit proximal tubular epithelial cell lines. *Kidney Int.* **42:**1130–1144

Satoh, T., Cohen, H.T., Katz, A.I. 1993. Different mechanisms of renal Na-K-ATPase regulation by protein kinases in proximal and distal nephron. *Am. J. Physiol.* **265:**F399–F405

Schwartz, J.H. 1995. Renal acid-base transport: the regulatory role of the inner medulary collecting duct. *Kidney Int.* **47:**333–341

Selvaggio, A.M., Schwartz, J.H., Bengele, H.H., Gordon, F.D., Alexander, E.A. 1988. Mechanisms of H^+ secreting by inner medullary collecting duct cells. *Am. J. Physiol.* **254:**F391–F400

Simmons, N.L. 1981. Stimulation of Cl^- secretion by exogenous ATP in cultured epithelial monolayers. *Biochim. Biophys. Acta* **646:**231–242

Simmons, N.L., Brown, C.D.A., Rugg, E.L. 1984. The action of epinephrine on Madin-Darby canine kidney cells. *Fed. Proc.* **43:**2225–2229

Takeda, M., Kobayashi, M., Endou, H. 1998. Establishment of a mouse clonal early proximal tubule cell line and outer medullary collecting duct cells expressing P_2 purinoceptors. *Biochem. Mol. Biol. Int.* **44:**657–664

Tauc, M., Gastineau, M., Poujeol, P. 1993. Toxin sensitivity of the calcium-dependent rubidium efflux in Madin-Darby canine kidney cells. *Biochem. Biophys. Res. Commun.* **190:**596–601

Tokuyama, Y., Hara, M., Jones, E.M., Fan, Z., Bell, G.I. 1995. Cloning of rat and mouse P_{2Y} purinoceptors. *Biochem. Biophys. Res. Commun.* **211:**211–218

Vojtek, A.B., Der, C.J. 1998. Increasing complexity of the ras signaling pathway. *J. Biol. Chem.* **273:**19925–19928

Webb, T.E., Henderson, D., King, B.F., Wang, S., Simon, J., Bateson, A.N., Burnstock, G., Barnard, E.A. 1996. A novel G protein-coupled P_2 purinoceptor (P2Y3) activated preferentially by nucleoside diphosphates. *Mol. Pharmacol.* **50:**258–265

Xing, M., Firestein, B.L., Shen, G.H., Insel, P.A. 1997. Dual role of protein kinase C in the regulation of $cPLA_2$-mediated arachidonic acid release by P_{2U} receptors in MDCK-D1 cells: involvement of MAP kinase-dependent and -independent pathways. *J. Clin. Invest.* **99:**805–814

Zegarra-Moran, O., Rommeo, G., Galietta, L.J.V. 1995. Regulation of transepithelial ion transport by two different purinoceptors in the apical membrane of canine kidney (MDCK) cells. *Br. J. Pharmacol.* **114:**1052–1056