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Complete inhibition of Na^+, K^+, Cl^- cotransport in Madin-Darby canine kidney cells by PMA-sensitive protein kinase

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Abstract

This study examines the involvement of hormones and neuromediators in the regulation of Na^+, K^+, Cl^- cotransport in renal epithelial cells using Madin-Darby canine kidney cells with low transepithelial electrical resistance ($194 \pm 47\,\Omega/cm^2$). In this cell line, Na^+, K^+, Cl^- cotransport measured as bumetanide-sensitive ^{86}Rb influx was inhibited up to 50–60% with agonists of P_2-purinoceptors (ATP ≈ ADP > UTP > AMP), slightly (15–30%) increased by activators of cAMP signaling (forskolin, 8-Br-cAMP) and was insensitive to activators of cGMP signaling (8-Br-cGMP, nitroprusside), EGF, angiotensin II, bradykinin, methacholine, propranolol, vasopressin, adenosine, dopamine and histamine. Thirty min of preincubation of MDCK cells with 0.1 μM PMA completely blocked the activity of Na^+, K^+, Cl^- cotransport whereas down-regulation of this enzyme by 24 h of preincubation with 1 μM PMA activated Na^+, K^+, Cl^- cotransport by 60% and abolished the effect of short-term treatment with PMA. Regulation of Na^+, K^+, Cl^- cotransport by ATP was insensitive to down-regulation of PMA-sensitive isoforms of protein kinase C. In addition, an inhibitor of protein kinase activity, staurosporine, abolished the effect of 0.1 μM PMA but did not change inhibition of this carrier by ATP. Thus, these results show for the first time that P_2-purinoceptors and PMA-sensitive isoforms of protein kinase C play a key role in the regulation of Na^+, K^+, Cl^- cotransport in MDCK cells. These results also show that neither PMA- nor staurosporine-sensitive forms of protein kinase are involved in the inhibition of Na^+, K^+, Cl^- cotransport by activators of P_2-purinoceptors. © 1998 Elsevier Science B.V.

Keywords: Na^+, K^+, Cl^- cotransport; Na^+, K^+ pump; P_2-purinoceptor; Protein kinase C; MDCK cell

1. Introduction

Twenty years ago, using microperfused tubule preparations, it was established that the diuretic effect of sulfamoylbenzoic acid derivates (SAD) is caused by inhibition of transcellular movement of salt and osmotically obliged water in the thick ascending limb of Henle's loop (THAL) [1,2]. Later on, it was shown that SAD-sensitive ion transporter is operated as electroneutral Na^+, K^+, Cl^- cotransport with variable stoichiometry and topology in different nephron segments (for recent data, see [3,4]). The control of

Abbreviations: MDCK, Madin-Darby canine kidney; PMA, 4β-phorbol 12-myristate 13-acetate; 4α-PMA, 4α-phorbol 12-myristate 13-acetate; SAD, sulfamoylbenzoic acid derivates; THAL, thick ascending limb of Henle's loop

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tubular reabsorption of salt and osmotically obliged water is also variable along tubular segments. Thus, in the collecting duct, cAMP directly increases the number of functioning water channels in apical membranes, the density of apical Na^+ channels and the open probability of 35 pS K^+ channels, whereas activation of protein kinase C-coupled signaling decreases the open probability of Na^+ channels and 35 pS ATP-sensitive K^+ channels (for review, see [5]). Little is known about the regulation of Na^+, K^+, Cl^- cotransport in the mammalian kidney. It has been shown that reabsorption of NaCl in the THAL is stimulated by cAMP signaling [6]. However, the relative contribution of Na^+, K^+, Cl^- cotransport and ion channels in this phenomenon is still a matter of controversy [7–9]. The present study examines the regulation of inward K^+ fluxes in epithelial cells derived from the canine kidney (MDCK). Our results indicate that in these cells, Na^+, K^+, Cl^- cotransport is completely inhibited by activation of protein kinase C with 4ß-phorbol 12-myristate 13-acetate (PMA), and partly by activation of P_2-purinoceptors. We also present evidence that inhibition of Na^+, K^+, Cl^- cotransport by P_2-purinoceptor agonists is not mediated by PMA-sensitive forms of protein kinase C.

2. Materials and methods

2.1. Cell culture

MDCK cells from the American Type Culture Collection (ATCC No. CCL 34) were used in this study. MDCK cells seeded on permeable support have a transepithelial resistance of $194 \pm 47\ \Omega/cm^2$. These results suggest that MDCK cells from our stock have the properties of MDCK-strain II described by Simmons [10] and clone C11 described by Gekle and co-workers [11]. In accordance with data presented in the last paper, this substrain of MDCK cells resembles intercalated cells of the collecting duct. MDCK cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and grown in MEM (Gibco Laboratories, Burlington, Canada), supplemented with sodium bicarbonate 2.5 g/l, HEPES 2 g/l, penicillin 100 U/ml, streptomycin 100 μg/ml and 10% fetal bovine serum (Wisent, WI). The medium was changed 2–3 times per week. The cells were passaged at subconfluent density by treatment with 0.05% trypsin (Gibco Laboratories) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline and scraped from the flasks with a rubber policeman. Cells were counted with a Coulter Counter. Dishes were inoculated at 1.25×10^3 cells/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 24-well plates, respectively.

2.2. Measurements of K^+ (^{86}Rb) influx

To study K^+ (^{86}Rb) influx, MDCK cells 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 and without agents indicated in the table and figures. Medium B contained: NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, D-glucose 5 mM, HEPES–tris 20 mM. Then, the preincubation medium was replaced with 0.25 ml of the same medium B with or without 1 mM ouabain and 20 μM bumetanide. The cells were incubated at 37°C for 5 min, and thereafter 0.25 ml of medium B containing 1–2 μCi/ml $^{86}RbCl$ was added. ^{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 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 (mg) and t is the incubation time (min). Protein content was measured by modified Lowry's method [12]. In the absence of ion transport inhibitors, the kinetics of ^{86}Rb uptake were linear up to at least 20 min (Fig. 1(A)). Based on these findings, an incubation time of 15 min was used to determine the initial rate of K^+ influx.

2.3. Chemicals

PMA, 4α-PMA, bradykinin, norepinephrine, isoproterenol, ATP, AMP, 8-Br-cAMP, forskolin, argi-

nine vasopressin, 8-Br-cGMP, ouabain, bumetanide, furosemide were purchased from Sigma (St. Louis, MO); dopamine, methacholine, phenylephrine, UTP, ADP, histamine from Research Biochemicals International (Natick, MA); angiotensin II from Armand-Frappier Institute (Laval, Canada); epidermal growth factor (EGF) from Gibco RBL (Gaithersburg, MD); ^{86}RbCl from Dupont (Boston, MA); salts, D-glucose and buffers were from Sigma and Anachemia (Montréal, Canada).

2.4. Statistical analysis

Experimental manipulations were performed in tri- or quadruplicate within any single experiment. The results are given as means ± standard errors. When appropriate, statistical significance was assessed by Student's *t*-test for unpaired data. Differences of $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

The Na^+, K^+ pump and Na^+, K^+, Cl^- cotransport, identified as ouabain-sensitive and ouabain-resistant bumetanide-sensitive ^{86}Rb influx, respectively, mediated ~65% and 30% of total K^+ influx in MDCK cells (Fig. 1(B)). Both the absolute values and relative activity of the Na^+, K^+ pump and Na^+, K^+, Cl^- cotransport were in accordance with previously-reported data [13–17]. In the presence of 10 μM bumetanide, 1 mM furosemide did not affect ^{86}Rb influx (data not shown). It is known that at this concentration, furosemide inhibits Na^+-independent K^+, Cl^- cotransport [18]. Thus, it may be concluded that (ouabain + bumetanide)-resistant K^+ influx, accounting for about 5% of ^{86}Rb influx (Fig. 1(B)), represents ion transport along its electrochemical gradient via ion channels and leakage of the MDCK membrane. We use the term ''passive permeability'' to combine these minor ion transport pathways.

Table 1 shows that an activator of protein kinase C, PMA, completely blocked Na^+, K^+, Cl^- cotransport in MDCK cells, whereas ATP inhibited this carrier by 50–60% ($p < 0.001$). Activation of cAMP signaling with 8-Br-cAMP, forskolin and cholera toxin increased the rate of Na^+, K^+, Cl^- cotransport by ~20%, 30% and 60%, respectively (Table 1), whereas activation of cGMP signaling with 8-Br-cGMP (1 mM) or nitroprusside (1 mM) did not affect Na^+, K^+, Cl^- cotransport (data not shown). Both

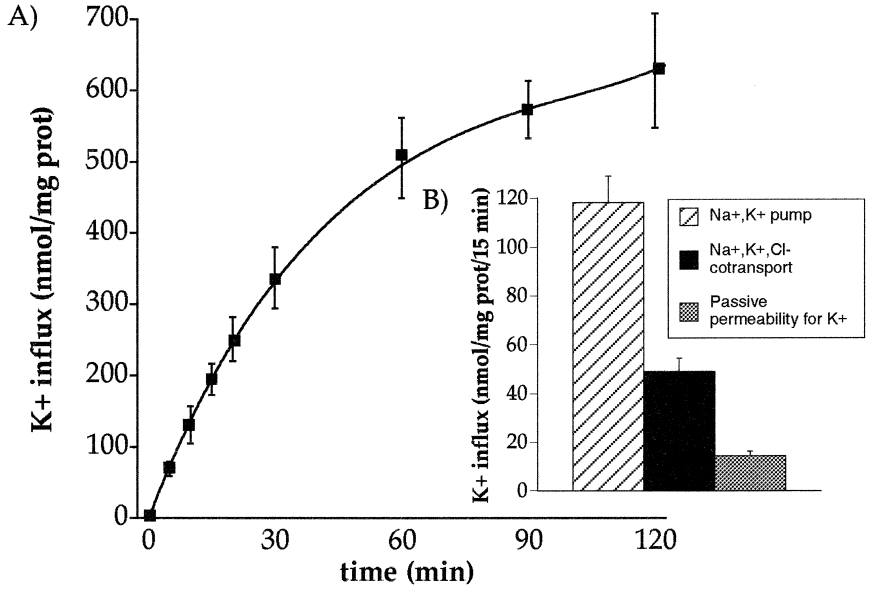

Fig. 1. Kinetics of K^+ (^{86}Rb) influx (A) and activity of Na^+, K^+ pump (ouabain-sensitive ^{86}Rb influx), Na^+, K^+, Cl^- cotransport (ouabain-resistant, bumetanide-sensitive ^{86}Rb influx) and passive permeability for K^+ (ouabain + bumetanide-resistant ^{86}Rb influx) (B) in MDCK cells. Means ± S.E. obtained in 2(A) and 20(B) experiments performed in quadruplicate or triplicate are given.

Table 1
Regulation of K^+ influx in MDCK cells by activators of cAMP signaling, protein kinase C and ATP

Addition	Na^+, K^+, Cl^- cotransport (%)	Na^+, K^+ pump (%)	Passive permeability for K^+ (%)
None (control)	100	100	100
Forskolin, 10 μM	129 ± 13 (9)	95 ± 11 (4)	93 ± 7 (9)
8-Br-cAMP, 1 mM	116 ± 9 (3)	119 ± 14 (1)	95 ± 6 (3)
Cholera toxin, 0.5 μg/ml	159 ± 11 (1) [a]	N.D.	98 ± 9 (1)
PMA, 0.1 μM	4 ± 7 (17) [d]	135 ± 9 (13) [b]	109 ± 6 (17)
4α-PMA, 0.1 μM	120 ± 8 (1)	N.D.	99 ± 4 (1)
ATP, 100 μM	43 ± 8 (11) [c]	130 ± 8 (5) [a]	103 ± 6 (11)

Cells were preincubated for 30 min with forskolin, 8-Br-cAMP, PMA, 4α-PMA, ATP or for 3 h with cholera toxin before the measurement of ion fluxes. Values of Na^+, K^+, Cl^- cotransport, Na^+, K^+ pump and passive permeability for K^+ in the absence of any addition (control) were taken as 100%. Means ± S.E. obtained in (n) experiments performed in quadruplicate are given.
[a] For $p < 0.05$.
[b] For $p < 0.02$.
[c] For $p < 0.001$.
[d] For $p < 0.0001$.

PMA and ATP activated the Na^+, K^+ pump by 20–40% ($p < 0.02$ and 0.05, respectively) (Table 1). We did not observe any significant effect of angiotensin II (1 μM), bradykinin (0.1 μM), vasopressin (0.1 μM), epidermal growth factor (1 μM), adenosine (100 μM), dopamine (100 μM), histamine (10 μM) and methacholine (100 μM) on K^+ influx in MDCK cells (data not shown). Previously, it was reported that in MDCK cells, PMA and dopamine inhibit Na^+, K^+-ATPase activity by 15–20% and 30–40%, respectively [15,19]. It should be emphasized, however, that crude membrane fractions of MDCK cells were used to estimate the activity of this enzyme in the above-cited studies.

Fig. 2 displays the dose-dependency of inhibition of Na^+, K^+, Cl^- cotransport by ATP, ADP, AMP and UTP. The rank-order of potency revealed in this study (ATP ~ ADP > UTP ≫ AMP) suggests that inhibition of Na^+, K^+, Cl^- cotransport by these compounds is mediated by P_{2X}- or P_{2Y}-purinoceptors [20].

The data listed below suggest that inhibition of Na^+, K^+, Cl^- cotransport by PMA is mediated via activation of protein kinase C. Indeed, as seen in Fig. 3, half-maximal inhibition of Na^+, K^+, Cl^- cotransport was observed at 10 nM of PMA, which is in accordance with data on the activation of protein kinase C by this compound in intact cells [21]. The inactive analogue of PMA, 4α-PMA, did not affect Na^+, K^+, Cl^- cotransport (Table 1). It was shown that 24 h preincubation of epithelial cells with 1 μM of PMA [22] or 3 μM of an analogue of PMA (PDBu) [23] causes down-regulation of protein kinase

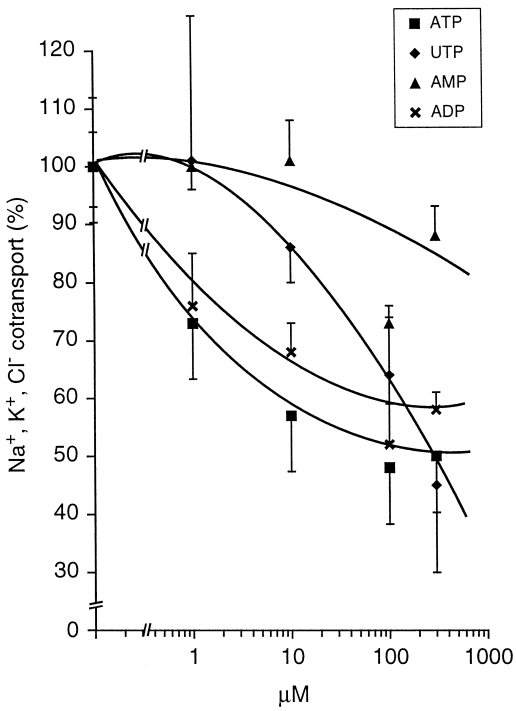

Fig. 2. Dose-dependence of Na^+, K^+, Cl^- cotransport on nucleotide concentrations. Cells were preincubated with nucleotide for 30 min before the measurement of ion fluxes. Values of Na^+, K^+, Cl^- cotransport in the absence of nucleotide were taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given.

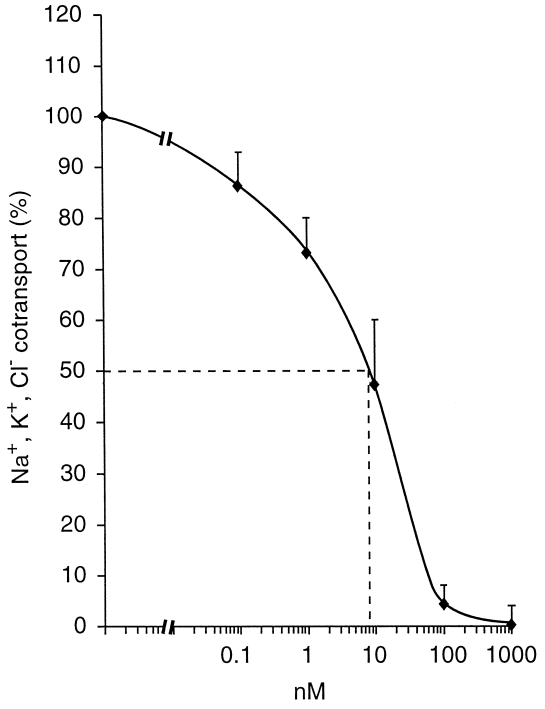

Fig. 3. Dose-dependence of Na^+, K^+, Cl^- cotransport on PMA. Cells were preincubated with PMA for 30 min before the measurement of ion fluxes. The value of Na^+, K^+, Cl^- cotransport in the absence of PMA was taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given.

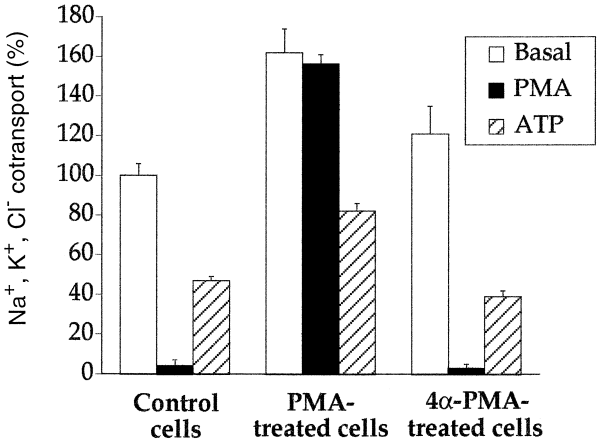

Fig. 4. Effect of down-regulation of protein kinase C activity on the regulation of Na^+, K^+, Cl^- cotransport by PMA and ATP. Cells were treated for 24 h with 1 μM of PMA or 4α-PMA in MEM containing 10% fetal calf serum. This medium was aspirated, cells were washed twice with medium A and incubated for 30 min in medium B with or without 0.1 μM PMA or 100 μM ATP. The value of Na^+, K^+, Cl^- cotransport in control cells without PMA or ATP (basal) was taken as 100%. Means ± S.E. obtained in experiments performed in quadruplicate are given.

C. In our study, treatment of MDCK cells with 1 μM PMA for 24 h increased Na^+, K^+, Cl^- cotransport by 50–70% (Fig. 4). This treatment completely abolished the inhibition of this carrier by 0.1 μM PMA (Fig. 4) which suggests the down-regulation of PMA-receptors. Neither the basal activity of Na^+, K^+, Cl^- cotransport nor its regulation by 0.1 μM PMA was affected by long-term treatment with 4α-PMA. A non-selective inhibitor of protein kinase activity, staurosporine ($K_{0.5}$ 7, 9, and 0.7 nM for purified protein kinase A, G, and C, respectively [24]), decreased the activity of Na^+, K^+, Cl^- cotransport by 33% (Fig. 5). In staurosporine-treated cells, PMA did not significantly affect the activity of this carrier. In contrast to the regulation by PMA, neither staurosporine nor down-regulation of protein kinase C affected the regulation of Na^+, K^+, Cl^- cotransport by ATP (Figs. 4 and 5).

Thus, data obtained in the present study demonstrate for the first time that, in epithelial cells of the mammalian kidney, Na^+, K^+, Cl^- cotransport could be completely inhibited by PMA-sensitive forms of protein kinase C and partly by agonists of P_2-purino-

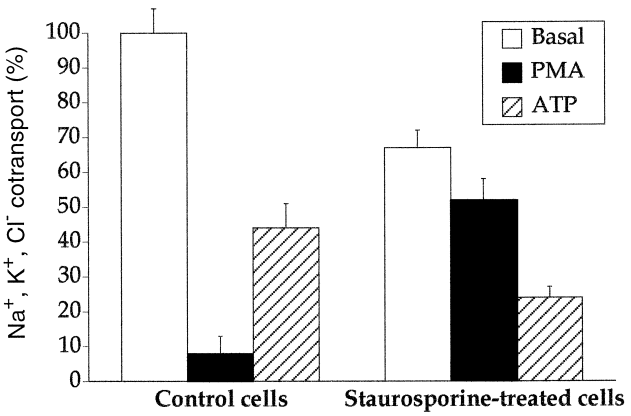

Fig. 5. Effect of staurosporine on the regulation of Na^+, K^+, Cl^- cotransport by PMA and ATP. Cells were washed twice with medium A and incubated for 30 min in medium B. This medium was then aspirated and cells were incubated for 10 min in 0.25 ml of medium B with or without 0.5 μM of staurosporine followed by the addition of 0.25 ml of medium B with or without 0.1 μM PMA or 100 μM ATP. Then, cells were incubated for 30 min before the measurement of ion fluxes. The value of Na^+, K^+, Cl^- cotransport in control cells without PMA, ATP or staurosporine (basal) was taken as 100%. Means ± S.E. obtained in experiments performed in triplicate are given.

ceptors. The regulatory properties of mammalian Na^+, K^+, Cl^- cotransport are extremely tissue specific. Thus, cAMP signaling activates Na^+, K^+, Cl^- cotransport in the human intestinal secretory epithelium [25], the shark rectal gland [26], SV-40 transformed cells derived from rabbit THAL [9], and fetal human non-pigmental ciliary epithelial cells [27]. In contrast, in the rat vascular smooth muscle [28,29], monkey retinal pigment epithelium [30] and human lymphocytes [31], cAMP inhibits this carrier. In rat erythrocytes [32] and bovine tracheal epithelial cells [33], SAD-sensitive ion fluxes are not affected by cAMP. It has been reported that cGMP activates Na^+, K^+, Cl^- cotransport in neuroblastoma NB-OK-1 cells [34], inhibits it in bovine endothelial cells [35,36] and parathyroid cells [37], and does not affect this carrier in rat erythrocytes and vascular smooth muscle cells [28,32]. We did not observe any effect of PMA on Na^+, K^+, Cl^- cotransport in rat erythrocytes and vascular smooth muscle cells [28,32]. In the rabbit tracheal epithelium, PMA led to 10-fold activation of SAD-sensitive Na^+ and Cl^- effluxes [38]. In bovine lens epithelial cells, Na^+, K^+, Cl^- cotransport was inhibited by PMA up to 50% [39]. However, in that study, cells were treated with 10 μM PMA for 2 h, whereas 1 h of treatment with 1 μM did not significantly modify the activity of the carrier. Thus, it is not clear whether the effect of 10 μM PMA was caused by activation of protein kinase C, inactivation of the enzyme due to down-regulation of its expression, or by a nonspecific action of the high PMA concentration.

To our knowledge, there are no data on the inhibition of Na^+, K^+, Cl^- cotransport by agonists of P_2-purinoceptors. Middleton et al. [40] previously reported 2-fold stimulation of Na^+, K^+, Cl^- cotransport in ATP-treated A6 cells. These results are opposed to our data with a 2–3-fold decrease of Na^+, K^+, Cl^- cotransport in ATP-treated MDCK cells (Fig. 2). However, we have to keep in mind that the A6 cell line is derived from the amphibian kidney, where renal anatomy and physiology are quite different from that of the mammalian nephron. Data obtained in the present study (Fig. 4) show that PMA-sensitive isoforms of protein kinase C are not involved in ATP-induced inhibition of Na^+, K^+, Cl^- cotransport. The role of GTP-binding proteins, cell volume, intracellular Ca^{2+} and monovalent ions in the regulation of Na^+, K^+, Cl^- cotransport by purinoceptors is currently being studied in our laboratory.

In terminals of the central and peripheral nervous systems, ATP and other P_2 agonists are colocalized with more specific neurotransmitters, such as catecholamines and acetylcholine. Local hypoxia and injury are another source of ATP release leading to purinergic activation [41]. We propose that P_2 receptor-mediated inhibition of Na^+, K^+, Cl^- cotransport, revealed for the first time in mammalian cells in this study, may be involved in the regulation of renal function under normal and pathophysiological conditions.

Screening the cDNA library obtained from different organs and species permitted cloning of 2 forms of Na^+, K^+, Cl^- cotransporters: NKCC1 and NKCC2. NKCC1 was revealed in all types of cells studied so far, whereas NKCC2 was selectively expressed in apical membranes of renal epithelial cells (for recent comparative analysis, see [42]). The site-specific effect of SAD on K^+ transport across the basolateral surface of the MDCK monolayer [13,43] indicates that the housekeeping NKCC1 is the major isoform expressed in these cells. Thus, it may be suggested that the lack of drastic inhibition of NKCC1 in other non-epithelial and epithelial cells by PMA and ATP compared with MDCK cells is caused by tubular segment-specific post-translation modification of NKCC1 or by a specific set of regulators which are involved in transduction of purinoceptor-induced signals to the transporter.

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