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ATP-Induced Inhibition of Na^+, K^+, Cl^- Cotransport in Madin-Darby Canine Kidney Cells: Lack of Involvement of Known Purinoceptor-Coupled Signaling Pathways

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Abstract. $P_{2U/2Y}$-receptors elicit multiple signaling in Madin-Darby canine kidney (MDCK) cells, including a transient increase of $[Ca^{2+}]_i$, activation of phospholipases C (PLC) and A_2 (PLA_2), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). This study examines the involvement of these signaling pathways in the inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells by ATP. The level of ATP-induced inhibition of this carrier (~50% of control values) was insensitive to cholera and pertussis toxins, to the PKC inhibitor calphostin C, to the cyclic nucleotide-dependent protein kinase inhibitors, H-89 and H-8 as well as to the inhibitor of serine-threonine type 1 and 2A phosphoprotein phosphatases okadaic acid. ATP led to a transient increase of $[Ca^{2+}]_i$ that was abolished by a chelator of $Ca^{2+}{}_i$, BAPTA. However, neither BAPTA nor the Ca^{2+} ionophore A231287, or an inhibitor of endoplasmic reticulum Ca^{2+}-pump, thapsigargin, modified ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. An inhibitor of PLC, U73122, and an inhibitor of MAPK kinase (MEK), PD98059, blocked ATP-induced inositol-1,4,5-triphosphate production and MAPK phosphorylation, respectively. However, these compounds did not modify the effect of ATP on Na^+,K^+,Cl^- cotransport activity. Inhibitors of PLA_2 ($AACOCF_3$), cycloxygenase (indomethacin) and lypoxygenase (NDGA) as well as exogenous arachidonic acid also did not affect ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. Inhibition of the carrier by ATP persisted in the presence of inhibitors of epithelial Na^+ channels (amiloride), Cl^- channels (NPPB) and Na^+/H^+ exchanger (EIPA) and was insensitive to cell volume modulation in anisosmotic media and to depletion of cells with monovalent ions, thus ruling out the role of other ion transporters in purinoceptor-induced inhibition of Na^+,K^+,Cl^- cotransport. Our data demonstrate that none of the known purinoceptor-stimulated signaling pathways mediate ATP-induced inhibition of Na^+,K^+,Cl^- cotransport and suggest the presence of a novel P_2-receptor-coupled signaling mechanism.

Key words: Na^+,K^+,Cl^- cotransport — P_2-purinoceptors — Intracellular signaling — MDCK cells

Introduction

The regulatory properties of the ubiquitous isoform of Na^+,K^+,Cl^- cotransport (NKCC1) are extremely tissue-specific. Thus, cyclic AMP activates Na^+,K^+,Cl^- cotransport in the human intestinal secretory epithelium (Matthews, Autrey & Madara, 1992), shark rectal gland (Lytle & Forbush, 1992), and fetal human nonpigmented ciliary epithelial cells (Crook & Polansky, 1994). By contrast, cAMP inhibits this carrier in rat vascular smooth muscle cells (Smith & Smith, 1987; Orlov et al., 1992), monkey retinal pigment epithelium (Kennedy, 1992) and human lymphocytes (Feldman, 1992), whereas it has no effect on Na^+,K^+,Cl^- cotransport in rat erythrocytes (Orlov et al., 1988) and bovine tracheal epithelial cells (Musch & Field, 1989). It has been reported that cGMP activates Na^+,K^+,Cl^- cotransport in neuroblastoma NB-OK-1 cells (Delporte et al., 1993), inhibits it in bovine endothelium (O'Donnell, 1989), HeLa cells (Kort & Koch, 1990) and parathyroid cells (DeFeo et al., 1991), and does not affect the carrier in rat erythrocytes and vascular smooth muscle (Orlov et al., 1988, 1992).

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In rat erythrocytes and vascular smooth muscle cells, an activator of protein kinase C (PKC)[1] 4β-phorbol 12-myristate 13-acetate (PMA), does not modulate Na^+,K^+,Cl^- cotransport (Orlov et al., 1988, 1992), whereas in the rabbit tracheal epithelium (Liedtke & Thomas, 1996) and NIH-3T3 fibroblasts (Hichami et al., 1996), PMA activates this ion carrier.

Madin-Darby canine kidney (MDCK) cells expressing NKCC1 (Delpire et al., 1994) are commonly used as a model of barrier epithelium (Lavelle et al., 1997). Recently, we have reported that in these cells, Na^+,K^+,Cl^- cotransport is insensitive to α-adrenergic, cholinergic and dopaminergic agonists as well as to vasopressin, bradykinin, angiotensin II and 8-Br-cGMP, but is completely blocked by activation of PKC with PMA and is partly inhibited by agonists of P_2-purinergic receptors (Gagnon et al., 1998). It is well documented that in monolayers of MDCK cells, activation of P_2-receptors is accompanied by augmented Cl^- secretion (Simmons, 1981) linked to rise of $[Ca^{2+}]_i$ (Paulmichl et al., 1991) and activation of phospholipase C (PLC) and A_2 (PLA_2) (Paulmichl et al., 1991; Firestein et al., 1992; Yang et al., 1997). Recently, it has been reported that in MDCK cells, P_2-receptor agonists also stimulate mitogen-activated protein kinase (MAPK) (Xing et al., 1997). In the present study, we examined the involvement of these signaling pathways in ATP-induced inhibition of Na^+,K^+,Cl^- cotransporter using a set of modulators of signal transduction shown in scheme presented in Discussion. We demonstrated that inhibition of Na^+,K^+,Cl^- cotransport by P_2-receptors in MDCK cells is not mediated by Gs/Gi GTP-binding proteins and is not related to elevation of $[Ca^{2+}]_i$ as well as to the activation of MAPK, PLA_2, polyphosphoinositide breakdown and staurosporine/calphostin-sensitive PKC.

Materials and Methods

Cell Culture

MDCK cells from the American Type Culture Collection (ATCC No. CCL 34, Rockville, MD) were used in these studies. They were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in MEM supplemented with 2.5 g/L sodium bicarbonate, 2 g/L HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco Laboratories, Burlington, Canada). The cells were passaged upon reaching subconfluent density by treatment with 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (Gibco Laboratories) and scarped 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 experiments (Figs. 3–5), cells were serum starved for 2 days in DMEM containing 0.1% BSA. MDCK cells seeded on permeable support had a transepithelial resistance of 201 ± 38 Ω/cm^2.

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

MDCK cells growing in 24-well plates were washed twice with 2 ml of medium A containing (in mM): 150 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 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 tested compounds. Medium B contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, HEPES-tris 20 (pH 7.4). The preincubation medium was then replaced with 0.25 ml of the same medium containing 1 mM ouabain with or without 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 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 modified Lowry's method (Hartee, 1972). As shown 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. Na^+,K^+,Cl^- cotransport was estimated as the rate of ouabain-resistant bumetanide-sensitive ^{86}Rb influx. The values of Na^+,K^+,Cl^- cotransport measured in the presence and absence of ouabain (46 ± 6 and 52 ± 9 nmol per mg prot per 15 min, respectively) were nor statistically different.

Intracellular Monovalent Ion Content

The intracellular content of Na^+, K^+ and Cl^- was determined as the distribution of isotopes between cells and extracellular medium under steady-state conditions (Orlov et al., 1996*a*). To adjust isotope equilibrium, MDCK cells were incubated for 2 hr in MEM containing 1 μCi/ml ^{86}Rb, ^{36}Cl or ^{22}Na, and for an additional 1 hr in medium B containing isotopes with the same specific radioactivity, with or without ouabain, PMA and ATP. To decrease the intracellular content of monovalent ions, cells were treated for 1 hr in a nonradioactive monovalent ion-depleted medium containing 300 mM sucrose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose and 20 mM HEPES-tris buffer (pH 7.4, 37°C) (for more details *see* Fig. 7). Aliquots of the incubation medium were then transferred into scintillation vials and the cells were washed 5 times with 2 ml of ice-cold medium C and lysed with SDS/EDTA mixture, as described above. Intracellular ion content (nmol/mg protein) was calculated as $V = A/am$ where A is the radioactivity of cell lysate (cpm), m is the protein content (mg) and a is the specific radioactivity of the incubation medium (cpm/nmol).

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

[1] Abbreviations: MAPK—mitogen-activated protein kinase; MDCK—Madin-Darby canine kidney; PKA and PKG—cAMP and cGMP-dependent protein kinases, respectively; PKC—protein kinase C; PLA_2 and PLC—phospholipase A_2 and C, respectively; PMA—4β-phorbol 12-myristate 13-acetate

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 3AM and 0.02% pluronic F-127 under permanent stirring. In a part of the samples, this medium also contained 50 μM BAPTA-AM (BAPTA-loaded cells). The cells were centrifuged (800 × g, 3 min), washed twice with medium B containing 1% BSA and 2.5 mM probenecid and then kept in 3 ml of the same medium at room temperature for no more than 3 hr. Before the measurement of fluorescence (F), 0.5 ml of cell suspension was centrifuged and the cells were washed with the medium B containing 1 mM probenecid, then resuspended in 2.5 ml of the same medium, F was measured at $\lambda_{ex} = 483$ nm and $\lambda_{em} = 523$ nm (slits 1 and 9 nm, respectively) using a SPEX FluoroMax spectrofluorimeter (Edison, NJ). Free intracellular Ca^{2+} concentration was measured 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-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, radioactive medium was aspirated, and cells were washed 3 times with 1 ml of medium A, followed by 30 min preincubation 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; 37°C). Thereafter, this medium was replaced with 0.25 ml of medium D for an additional 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 the addition of SDS/EDTA mixture. Cell lysates were applied to column containing 0.5 g DOWEX-AG 1-X8 (formate form), and inositol-3-phosphate was resolved as described previously (Orlov et al., 1992).

MAPK Phosphorylation

Serum-starved cells grown in 6-well plates were stimulated with desired agonists for 10 min, washed twice with ice-cold phosphate-buffered saline and lysed in 150 μl of the 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 leupetin, 1 μg/ml aprotinin, 200 μM Na-orthovanadate and 1 mM NaF. The lysed cells were scarped and centrifuged at 14,000 rpm for 20 min in a microcentrifuge. A 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 p42/p44 MAPK was determined by western blot analysis with antibodies against phospho-ERK following the manufacturer's instructions and by documenting the electrophoretic mobility shift of phosphorylated MAPK, using anti-p42 ERK antibodies.

Chemicals

PMA, ATP, ouabain, bumetanide, amiloride, probenecid, indomethacin, NDGA (nordihydroguaiaretic acid), arachidonic acid, DOWEX-AG 1-X8, Sigma (St. Louis, MO); EIPA (ethylisopropylamiloride), okadaic acid, NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid, Research Biochemicals International (Natick, MA); cholera toxin, pertussis, toxin, thapsigargin, A23187, BAPTA-AM, staurosporine, calphostin C, H-8, H-89, $AACOCF_3$, U-73122, Calbiochem (La Jolla, CA); fluo 3AM, pluronic F-127, Molecular Probes (Eugene, OR); PD98059 and phospho-ERK antibodies, New England Biolab, (Beverly, MA); ^{86}RbCl, ^{22}NaCl, H^{36}Cl, *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).

Table 1. Effect of cholera and pertussis toxins on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells

Preincubation with toxins	Na^+,K^+,Cl^- cotransport, %		
	Control	PMA	ATP
None (control)	100 ± 6	4 ± 3 (96)*	57 ± 4 (43*
Cholera toxin	155 ± 17#	8 ± 5 (95)*	78 ± 9 (50)*
Pertussis toxin	116 ± 11	6 ± 4 (95)*	61 ± 4 (47)*

Cells were preincubated with or without 0.5 μg/ml cholera or pertussis toxins for 3 hr, followed by stimulation with 0.1 μM PMA or 100 μM ATP for 30 min in medium B. This medium was then aspirated, and 0.25 ml of medium B containing 1 mM ouabain with or without 20 μM bumetanide, 0.1 μM PMA or 100 μM ATP 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^+,Cl^- cotransport in toxin-untreated cells without PMA or ATP was taken as 100%. Means ± SE obtained in experiment performed in quadruplicate are given. The percentages of inhibition of Na^+,K^+,Cl^- cotransport by PMA and ATP are shown in parentheses. #$P < 0.05$ as compared with toxin-untreated cells; *$P < 0.005$ as compared with PMA- and ATP-untreated cells.

Results

Role of Cholera and Pertussis Toxin-Sensitive GTP-binding Proteins

To evaluate the role of Gp in purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport, we examined the effect of exogeneous ATP on this carrier in the presence of cholera and pertussis toxins. Previously, it has been shown that ADP ribosylation of Gi by pertussis toxin blocks ATP-induced inositol 1,4,5-triphosphate ($InsP_3$) production in MDCK cells (Paulmichl et al., 1991; Yang et al., 1997), whereas cholera toxin-sensitive Gs are involved in cAMP production triggered by ATP-induced PGE_2 release (Post et al., 1996). As shown in Table 1, treatment of MDCK cells with cholera toxin augmented Na^+,K^+,Cl^- cotransport by ~50%, which is consistent with a moderate elevation of its activity in MDCK cells treated with a direct activator of adenylate cyclase, forskolin (Gagnon et al., 1998). In contrast to cholera toxin, pertussis toxin did not affect basal Na^+,K^+,Cl^- cotransport activity. Neither cholera toxin nor pertussis toxin modified the level of inhibition of Na^+,K^+,Cl^- cotransport by ATP (40–50%) and the complete inhibition of the carrier by PMA (Table 1). The same level of inhibition of bumetanide-sensitive ^{86}Rb influx by ATP and PMA

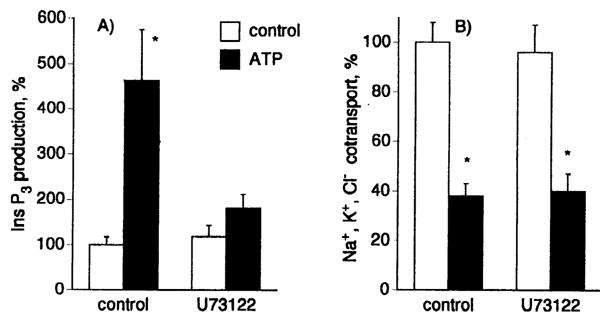


Fig. 1. Effect of U73122 on ATP-induced inositol-1,4,5-triphosphate ($InsP_3$) production (*A*) and inhibition of Na^+,K^+,Cl^- cotransport (*B*) in MDCK cells. Cells were preincubated with or without 10 μM U73122 for 30 min, followed by stimulation with 100 μM ATP for 5 min (*A*) or 30 min (*B*). $InsP_3$ production and Na^+,K^+,Cl^- cotransport values in the absence of ATP were taken as 100%. Means ± SE obtained in experiments performed in quadruplicate are given. $*P < 0.01$ as compared to ATP-untreated cells.

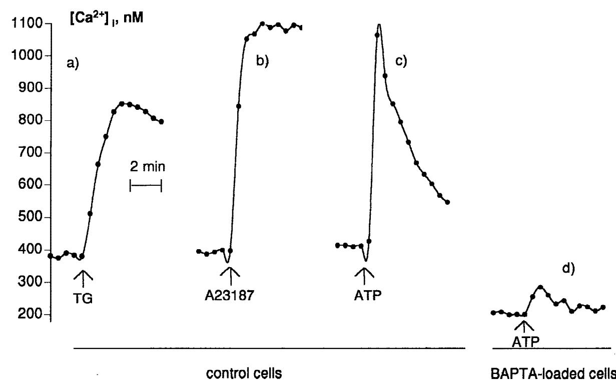


Fig. 2. Effect of thapsigargin (TG), A23187 and ATP on intracellular free calcium concentration ($[Ca^{2+}]_i$) in control and BAPTA-loaded MDCK cells. $[Ca^{2+}]_i$) was measured as described in Methods after stimulation of intact MDCK cells (*a–c*) or BAPTA-loaded MDCK cells (*d*) with 0.25 μM thapsigargin (*a*), 3 μM A23187 (*b*), or 100 μM ATP (*c* and *d*).

was observed in the absence of ouabain (*data not shown*).

Role of Polyphosphoinositide Breakdown

Incubation of MDCK cells with ATP resulted in a 4–5 fold increase of $InsP_3$ production (Fig. 1*A*), which is in accordance with previously reported data (Paulmichl et al., 1991). Preincubation of MDCK cells with an inhibitor of PLC, U73122, attenuated ATP-induced $InsP_3$ release by 90%. However, the same treatment with U73122 failed to alter the effect of ATP on Na^+,K^+,Cl^- cotransport activity (Fig. 1*B*).

Role of $Ca^{2+}{}_i$

It is well documented that activation of P_2-receptors increases $[Ca^{2+}]_i$ in MDCK cells (Paulmichl et al., 1991; Delles, Haller & Dietl, 1995). To evaluate the role of $[Ca^{2+}]_i$ in the regulation of Na^+,K^+,Cl^- cotransport, the effect of ATP and PMA on the carrier activity was measured in the presence of Ca^{2+}-modulating agents: an intracellular Ca^{2+} chelator, BAPTA, an inhibitor of endoplasmic reticulum Ca^{2+} pump, thapsigargin, and the Ca^{2+}-ionophore, A23187. As shown in Fig. 2, thapsigargin, A23187 and ATP led to rise of $[Ca^{2+}]_i$ up-to 500–700 nM over control values. Preincubation of MDCK cells with BAPTA-AM decreased the basal $[Ca^{2+}]_i$ and dramatically inhibited the ATP-induced $[Ca^{2+}]_i$ response (Fig. 2). However, none of the $[Ca^{2+}]_i$-modulating compounds had any significant effect on basal Na^+,K^+,Cl^- cotransport and on its inhibition by ATP and PMA (Table 2).

Table 2. Regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells in the presence of Ca^{2+}-modulating compounds

Additions, μM	Na^+,K^+,Cl^- cotransport, %		
	Control	PMA	ATP
None (control)	100 ± 9	2 ± 4*	47 ± 6*
BAPTA-AM, 50	97 ± 11	3 ± 2*	39 ± 4*
Thapsigargin, 0.25	94 ± 7	4 ± 6*	45 ± 7*
A23187, 3	129 ± 17	3 ± 1*	36 ± 6*

Cells were preincubated in medium B with or without 0.2% BSA and BAPTA-AM for 30 min. Thereafter, the medium was aspirated and the cells were incubated for an additional 30 min with or without thapsigargin, A23187, 0.1 μM PMA and 100 μM ATP. This medium was them aspirated, and 0.25 ml of the same media 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 $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± SE, obtained in experiment performed in quadruplicate are given. $P < 0.005$ as compared with PMA- and ATP-untreated cells.

Role of Serine-Threonine Protein Kinases and Phosphoprotein Phosphatases

To investigate the role of serine-threonine phosphorylation in the regulation of Na^+,K^+,Cl^- cotransport by ATP, we first applied different inhibitors of serine-threonine protein kinases and phosphatases. Table 3 shows that the nonspecific inhibitor of protein kinases, staurosporine (Hidaka et al., 1984), decreased basal Na^+,K^+,Cl^- cotransport by ~25%, drastically attenuated the effect of PKC activator, PMA, and had somewhat of an impact on ATP-induced inhibition of Na^+,K^+,Cl^- cotransport. The selective inhibitor of PKC calphostin C (Kobayashi et al., 1989) enhanced the activity of the carrier by 40% and

Table 3. Effect of staurosporine, calphostin C, H-89, H-8 and okadaic acid on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells

Additions, μM	Na^+,K^+,Cl^- cotransport, %		
	Control	PMA	ATP
None (control)	100 ± 6	6 ± 4 (94)*	45 ± 7 (55)*
Staurosporine, 0.25	73 ± 6	54 ± 8 (22)	24 ± 3 (67)*
Calphostin C, 0.12	139 ± 11	135 ± 12 (3)	62 ± 6 (55)*
H-89, 20	104 ± 9	5 ± 2 (95)*	42 ± 7 (59)*
H-8, 20	96 ± 6	3 ± 2 (97)*	44 ± 8 (54)*
Okadaic acid, 1	229 ± 20#	6 ± 1 (97)*	57 ± 8 (75)*

Cells were preincubated in medium B with or without compounds listed in the left column, followed by 30 min of incubation in the same media with or without 0.1 μM PMA and 100 μM ATP. This medium was then aspirated, and 0.25 ml of the same media 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 $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport in the absence of any additions was taken as 100%. Means ± SE obtained in 3 experiments performed in quadruplicate are given. The percentages of inhibition of Na^+,K^+,Cl^- cotransport by PMA and ATP are shown in parentheses. $*P < 0.001$ as compared with control cells; $*P < 0.001$ as compared with PMA- and ATP-untreated cells.

completely abolished the effect of PMA. However, calphostin C had no significant influence on the level of ATP-induced inhibition of Na^+,K^+,Cl^- cotransport (Table 3). The selective inhibitors of cAMP- and cGMP-dependent protein kinases (PKA and PKG), H-89 and H-8, respectively (Chijiwa et al., 1990) did not influence basal Na^+,K^+,Cl^- cotransport activity and its modulation by PMA and ATP. Interestingly, the inhibitor of serine-threonine phosphatases type 1 and 2A (PP1/PP2A) okadaic acid (Cohen, Holmes & Tsukitani, 1990) enhanced Na^+,K^+,Cl^- cotransport by more than 2-fold, but did not alter regulation of the carrier by PMA and ATP (Table 3). These data suggest that despite the ability of PKC and PP1/PP2A to regulate Na^+,K^+,Cl^- cotransport, these enzymes as well as PKA and PKG are not involved in the inhibition of the carrier by ATP.

Role of MAP Kinase

In MDCK cells, both ATP and PMA induced MAPK phosphorylation, as determined by immunoblotting of cell lysate with anti-phospho-ERK antibodies (Fig. 3*B*) and by documenting the electrophoretic mobility shift of the p42 isoform of ERK (Fig. 3*A*). It should be mentioned that, in contrast to vascular smooth muscle cells, calf serum only slightly potentiated MAPK phosphorylation in MDCK cells (Fig. 3*A* and *B*). This peculiarity of MDCK cells is probably caused by their relatively low abundance of receptors to growth factor and lysophosphatidic acid, the major components involved in the activation of MAPK in vascular smooth muscle (Force & Bonventre, 1998). In contrast to ATP and PMA, calf serum did not inhibit Na^+,K^+,Cl^- cotransport in MDCK cells (Fig. 3*C*). Figure 4*A* shows that phosphorylation of p42/p44 ERK by ATP was abolished in the presence of an inhibitor of MAPK kinase (MEK), PD98059. However, this compound did not alter the basal Na^+,K^+,Cl^- cotransport or its inhibition by ATP (Fig. 4*B*).

As mentioned above, modulation of Ca^{2+}_i homeostasis with BAPTA and thapsigargin does not affect inhibition of Na^+,K^+,Cl^- cotransport by ATP (Table 2). Recently, we reported that purinoceptor-induced inhibition of this carrier is also insensitive to PKC downregulation under longterm treatment with PMA (Gagnon et al., 1998). To further examine the role of MAPK in ATP-dependent regulation of Na^+,K^+,Cl^- cotransport, we compared the effect of BAPTA and longterm exposure to PMA on ATP-induced p42/p44 ERK phosphorylation, using PMA-, thapsigargin- and epidermal growth factor (EGF)-treated cells as controls. Figure 5 shows that, in contrast to regulation of Na^+,K^+,Cl^- cotransport, both BAPTA and PKC downregulation decreased ATP-induced MAPK phosphorylation indicating that this signaling pathway is mediated by Ca^{2+}- and diacyl glycerol-sensitive isoforms of PKC. BAPTA partially reduced PMA-induced p42/p44 phosphorylation, whereas PKC downregulation completely abolished the effect of acute exposure to PMA. Both BAPTA and PKC downregulation completely abolished thapsigargin-induced p42/p44 phosphorylation. In contrast to ATP, PMA, and thapsigargin, the slight increment of MAPK phosphorylation induced by EGF was insensitive to downregulation of PKC and Ca^{2+}_i-chelation with BAPTA (Fig. 5). The last observation is consistent with data on the Ca^{2+}_i- and PKC-independent mechanism of ERK phosphorylation by agonists of receptor tyrosine kinases (Force & Bonventre, 1988).

Role PLA_2-Derived Products

It has been documented that PMA as well as P_{2Y} and P_{2U} receptor agonists stimulate PLA_2 in MDCK cells, resulting in the production of arachidonic acid followed by PGE_2 release (Parker, Daniel & Waite, 1987; Firestein et al., 1996). In monolayers of MDCK cells, PGE_2 mimicked the effect of ATP on short current and Cl^- secretion (Simmons, 1991; Steidl et al., 1991), whereas an active analogue of arachidonic acid, $AACOCF_3$ blocked the release of arachidonic acid from ATP-treated cells (Firestein et al., 1996). Therefore, we investigated a possible role of arachidonic acid and/or its metabolites in the regulation of Na^+,K^+,Cl^- cotransport by ATP. Neither the addition of exogenous arachidonic acid (10 μM) nor the inhibition of PLA_2 by $AACOCF_3$ (25 μM), of

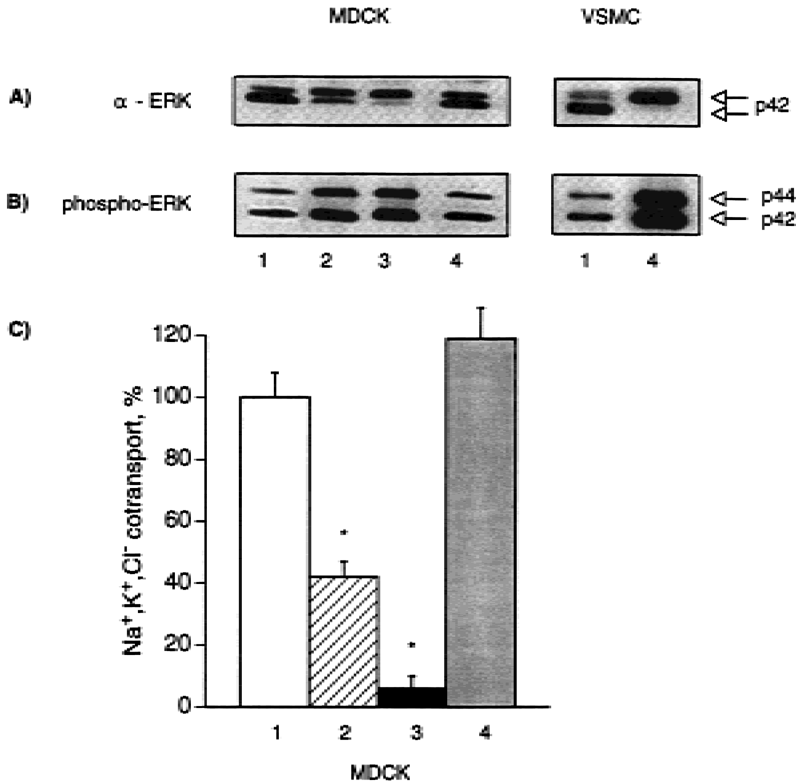


Fig. 3. Effect of ATP, PMA and calf serum on phosphorylation of MAP kinase (ERK) (*A* and *B*) and the activity of Na^+,K^+,Cl^- cotransport (*C*). MDCK cells or vascular smooth cells (VSMC) were serum starved for 2 days in DMEM containing 0.1% BSA, followed by incubation in medium B (control—1) or in medium B with 100 μM ATP (2), 0.1 μM PMA (3) or 10% calf serum (4) for 10 min (*A* and *B*), or 30 min (*C*). Phosphorylation of MAPK was determined by gel retardation of p42 using anti-p42 ERK antibodies (*A*), or by immunoblotting with phospho-specific anti-p42/p44 ERK antibodies (*B*). VSMC were obtained from rat aorta as described previously in details (Orlov et al., 1996*b*) and were cultured similarly to MDCK cells. Means ± SE obtained in experiments performed in quadruplicate are given. $*P < 0.001$ as compared with the controls.

prostaglandin synthase by indomethacin (10 μM) and of cycloxygenase by NDGA (10 μM) affected basal activity of Na^+,K^+,Cl^- cotransport as well as the regulation of this carrier by PMA and ATP (*data as shown*). This suggests that PLA_2 does not mediate the effect of ATP on Na^+,K^+,Cl^- cotransport.

Role of Intracellular Monovalent Ions and Cell Volume

At the basolateral membrane of MDCK cells, Na^+,K^+,Cl^- cotransport operates in parallel to the Na^+,K^+ pump, Na^+/H^+ exchange and intermediate conductance K^+ (26 pS) and Cl^- (46 pS) channels. Both intermediate conductance and Ca^{2+}-activated maxi-K^+ channels (220 pS) are expressed in the apical membrane of MDCK cells. The apical membrane is also highly abundant with maxi-Cl^- channels (460 pS), whereas the content of Na^+ channels is rather low (for review *see* Lang & Paulmichl, 1995). It is well documented that intracellular Cl^- negatively regulates Na^+,K^+,Cl^- cotransport (Haas, 1994). In addition, Breitwiezer with coworkers (1996) reported that in squid axon this carrier is also inhibited by intracellular Na^+. Apart from direct regulation of Na^+,K^+,Cl^- cotransport, modulation of the content of intracellular monovalent ions can alter MDCK cell volume, thus also affecting volume-sensitive Na^+,K^+,Cl^- cotransporter (Haas, 1994). Considering this, it may be suggested that the regulation of bumetanide-sensitive K^+ influx by ATP is mediated by modulation of the activity of monovalent ion transporters distinct from Na^+,K^+,Cl^- cotransport. To test this hypothesis, we studied the effect of inhibitors of the above-mentioned ion transporters on the regulation of Na^+,K^+,Cl^- cotransport and modulation of the context of intracellular monovalent ions by ATP and PMA.

We did not observe any effect of a potent inhibitor of epithelial Na^+ channels, amiloride (20 μM), a putative Cl^- channel blocker, NPPB (100 μM), and an inhibitor of Na^+/H^+ exchanger, EIPA (20 μM), on the regulation of Na^+,K^+,Cl^- cotransport by ATP and PMA (*data not shown*). It is well documented that at a concentration of 3–5 mM Ba^{2+} completely blocks ion transport across different type of K^+ channels, including Ca^{2+}-activated maxi-K^+ channels in MDCK cells (Tauc et al., 1993). Surprisingly, we revealed that $BaCl_2$ inhibited basal activity of Na^+,K^+,Cl^- cotransport with an IC_{50} of ~1 mM (Fig. 6, curve 1). In contrast to Na^+,K^+,Cl^- cotransport, (ouabain + bumetanide)-resistant ^{86}Rb influx termed here as a passive permeability for K^+ was not significantly affected by Ba^{2+} (Fig. 6, curve 2), indicating that the contribution of Ba^{2+}-sensitive K^+ channels to K^+ influx in MDCK cells under basal condition is negligible.

Table 4 shows that both ATP and PMA slightly increase K^+_i content and decreased Na^+_i content, which is probably due to ~30% activation of Na^+,K^+ pump in

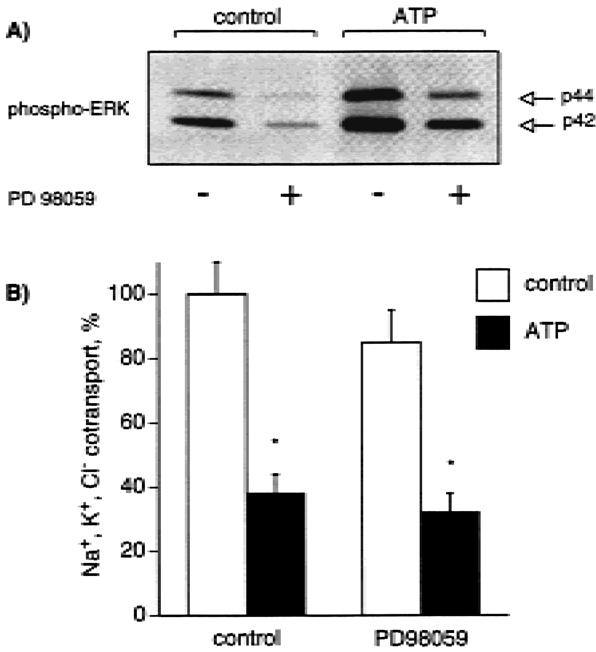


Fig. 4. Effect of PD98059 on ATP-induced MAP kinase phosphorylation (*A*) and Na^+,K^+,Cl^- cotransport inhibition (*B*). Serum-starved MDCK cells were preincubated in medium B with or without 50 μM PD98059 for 1 hr, followed by stimulation with 100 μM ATP for 10 min (*A*) or 30 min (*B*) as indicated. MAPK phosphorylation was determined by immunoblotting with phosphospecific anti-p42/p44 ERK antibodies. Means ± SE obtained in experiments performed in quadruplicate are given. $*P < 0.01$ as compared with the controls.

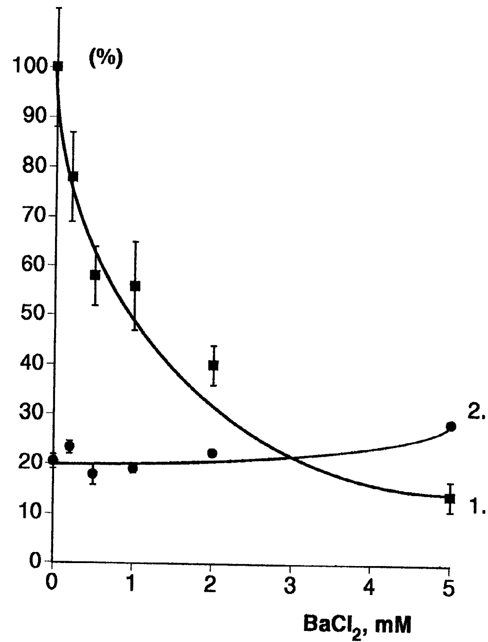


Fig. 6. Na^+,K^+,Cl^- cotransport activity and passive permeability for K^+ in MDCK cells preincubated with different $BaCl_2$ concentration for 30 min, followed by measurement of ouabain-resistant bumetanide-sensitive ^{86}Rb influx (Na^+,K^+,Cl^- cotransport activity—curve 1), or (ouabain + bumetanide)-resistant ^{86}Rb influx (passive permeability for K^+—curve 2). Na^+,K^+,Cl^- cotransport values in the absence of $BaCl_2$ were taken as 100%. Means ± SE obtained in experiment performed in quadruplicate are given.

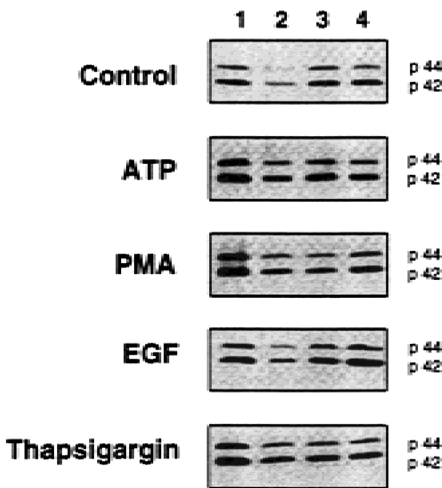


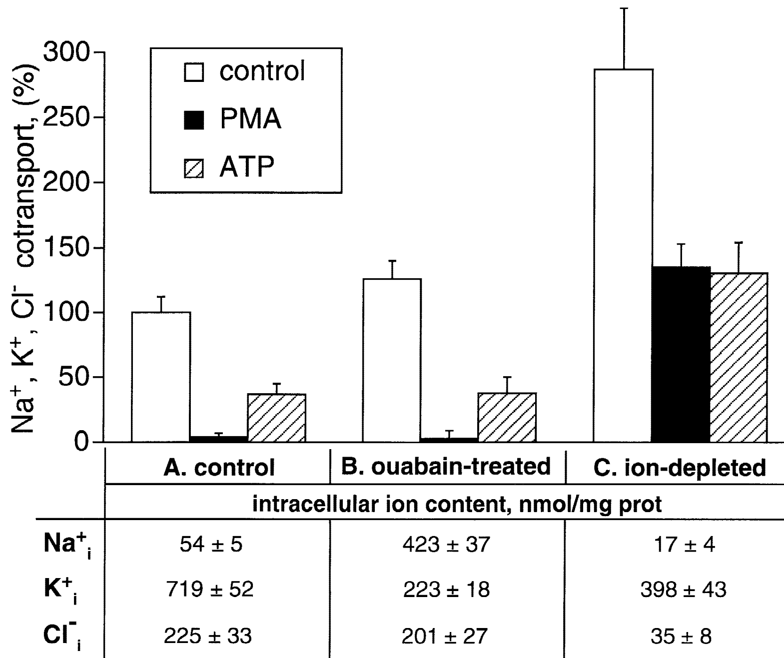
Fig. 5. Effect of MAPK kinase inhibition by PD98059, downregulation of PKC under long-term treatment with PMA and intracellular calcium depletion with BAPTA on MAP kinase phosphorylation induced by ATP, PMA, EGF or thapsigargin. Serum-starved cells were preincubated in DMEM containing 0.1% BSA with vehicle (1) or with 50 μM PD98059 for 1 hr (2), 1 μM PMA for 24 hr (3), or 50 μM BAPTA-AM for 1 hr (4), followed by stimulation with vehicle (control), 100 μM ATP, 0.1 μM PMA, 50 ng/ml EGF or 0.5 μM thapsigargin for 10 min. MAPK phosphorylation was determined by immunoblotting with phosphospecific antip42/p44 ERK antibodies.

Table 4. The content of monovalent ions in control and PMA- or ATP-treated MDCK cells

Additions, μM	Intracellular ion content, nmol/mg prot		
	K^+	Na^+	Cl^-
None (control)	719 ± 52	54 ± 5	225 ± 33
PMA, 0.1	825 ± 48	45 ± 6	236 ± 47
ATP, 100	788 ± 56	42 ± 4	240 ± 31

Cells were incubated for 2 hr in DMEM containing 1 μCi/ml ^{86}Rb, ^{22}Na or ^{36}Cl and for an additional 1 hr in medium B containing isotopes with the same specific radioactivity and with or without PMA or ATP. For more details see Materials and Methods. Means ± SE obtained in experiment performed in quadruplicate are given.

PMA- and ATP-treated MDCK cells (Gagnon et al., 1998). Neither PMA nor ATP affected Cl^-_i content (Table 4). To further examine the role of intracellular monovalent ions in purinergic regulation of Na^+,K^+,Cl^- cotransport, we pretreated cells with an inhibitor of Na^+,K+ pump, ouabain, and with monovalent ion depleted medium. Figure 7 shows that 1 hr-treatment of MDCK cells with ouabain led to 8-fold increase of Na^+_i content and decreased K^+_i by 3-fold (column B). However, neither basal activity of Na^+,K^+,Cl^- cotransport nor



	A. control	B. ouabain-treated	C. ion-depleted
	intracellular ion content, nmol/mg prot		
Na^+_i	54 ± 5	423 ± 37	17 ± 4
K^+_i	719 ± 52	223 ± 18	398 ± 43
Cl^-_i	225 ± 33	201 ± 27	35 ± 8

Fig. 7. Effect of intracellular monovalent ions on the regulation of Na^+,K^+,Cl^- cotransport by PMA and ATP in MDCK cells preincubated in medium B (*see* Materials and Methods) for 30 min, followed by incubation with the same medium (*A*—control), medium B, containing 1 mM ouabain (*B*—ouabain-treated cells) or in monovalent ion-depleted medium (*C*—ion-depleted cells), containing 300 mM sucrose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM HEPES-tris (pH 7.4; 37°C). After 30 min, these media were replaced by the same media with or without 0.1 μM PMA or 100 μM ATP for an additional 30 min. The cells were then transferred onto ice, the media were aspirated, and 0.25 ml of medium B containing 1 mM ouabain with or without 20 μM bumetanide, 0.1 μM PMA or 100 μM ATP was added. ^{86}Rb uptake was triggered by the addition of 0.25 ml prewarmed medium B with 1 μCi/ml $^{86}RbCl$ and terminated after 5 min incubation at 37°C by the addition of ice-cold medium C (*see* Materials and Methods). The values of Na^+,K^+,Cl^- cotransport in control cells (*A*) without PMA and ATP were taken as 100%. The intracellular concentration of exchangeable Na^+, K^+ and Cl^- in control (*A*), ouabain-treated (*B*) and monovalent ion-depleted (*C*) cells was measured as described in Materials and Methods and is shown below the columns. Means ± SE obtained in 2 experiments performed in quadruplicate are given.

its regulation by PMA and ATP was affected in ouabain-treated cells. Pretreatment of MDCK cells with monovalent ion-depleted medium decreased intracellular Na^+, K^+ and Cl^- content by 3-, 2- and 7-fold respectively (Fig. 7, column C). In these cells, basal Na^+,K^+,Cl^- cotransport was increased by 2-3-fold, which is in accordance with the data on the feedback regulation of this carrier by Cl^-_i (Haas, 1994). Under these conditions, PMA inhibited Na^+,K^+,Cl^- cotransport by 50% only, whereas the level of inhibition of the carrier by ATP was unchanged.

The relatively low permeability of MDCK cells for water and [^{14}C]-urea (Lavelle et al., 1997), commonly used as a marker of intracellular water space, complicates the study of the effect of PMA and ATP on cell volume. However, the lack of a significant effect of these compounds on the total content of intracellular ions (Table 4), i.e., of major intracellular osmolites, suggests that they did not lead to essential cell volume perturbation. To examine whether or not cell volume alteration can modify the sensitivity of Na^+,K^+,Cl^- cotransport to PMA and ATP, we measured the activity of this carrier in unisosmotic media. The shrinkage of MDCK cells under elevation of osmolality of the incubation medium from 200 to 480 mosm led to ~10-fold activation of Na^+,K^+,Cl^- cotransport (Fig. 8), which is in accordance with data obtained for the majority of cells expressing NKCC1 isoform of the carrier (Haas, 1994). However, independently of cell volume, PMA still completely inhibited this ion transporter whereas ATP decreased its activity by 50–60%.

Discussion

During the last decade, it was shown that P_2-purinoceptors affect the function of MDCK cells utilizing multiple signaling pathways, including activation of Gi/Gq proteins followed by elevation of the activity of PLC, PLA_2, adenylate cyclase, PKC and MAPK (Fig. 9). Data obtained in the present study indicate that none of the above-mentioned signaling pathways are involved in recently discovered purinoceptor-induced inhibition of Na^+,K^+,Cl^- cotransport (Gagnon et al., 1998).

The rank-order of potency of agonists of purinoceptors as inhibitors of Na^+,K^+,Cl^- cotransport in MDCK cells (ATP~ADP > UTP >> AMP) (Gagnon et al., 1998) suggest that their effect is mediated by P_{2X}- or P_{2Y}-receptors[2]. Indeed, in contrast to P_{2X} and P_{2Y}, the P_{2U} subtype exhibits the highest sensitivity to UTP, whereas the P_{2Z} subtype is completely insensitive to ADP and AMP. The P_{2T} subtype is activated by ADP and an-

[2] The apparent affinity of P_2-receptors for ATP can be affected by the partial hydrolysis of this compound by ecto-ATPases. This hypothesis is currently examined in our laboratory.

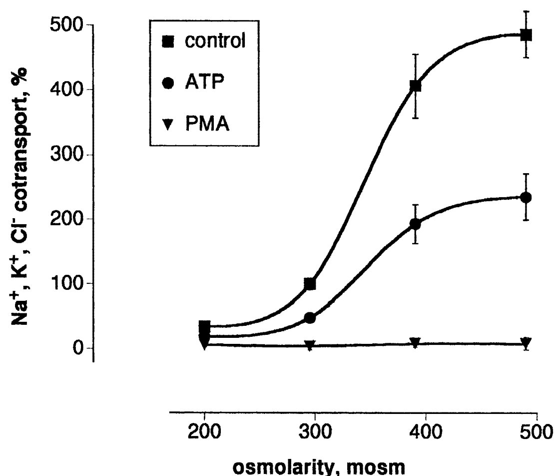


Fig. 8. Dependence of Na^+,K^+,Cl^- cotransport in MDCK cells on osmolality of the incubation medium. Cells were preincubated for 1 hr in medium B (*see* Materials and Methods), followed by incubation with 0.1 μM PMA or 100 μM ATP in the same medium. After 30 min of stimulation, this medium was aspirated, and 0.25 ml of medium B containing 1 mM ouabain with or without 20 μM bumetanide, 0.1 μM PMA or 100 μM ATP was added. After 5 min, ^{85}Rb uptake was initiated by the addition of 0.25 ml of medium B with 1 μCi/ml ^{85}RbCl. The final osmolality of the medium was decreased to 200 mosm and increased to 490 mosm by lowering NaCl concentration and by addition of sucrose to ^{86}Rb-containing medium, respectively. The final osmolality values was measured with a Knauer milliosmometer (Berlin, Germany). The values of Na^+,K^+,Cl^- cotransport in PMA- and ATP-untreated cells at 295 mosm were taken as 100%. Means ± SE obtained in 2 experiments performed in quadruplicate are given.

tagonized by ATP and AMP and appears to be exclusively expressed in platelets (Dubyak & El-Moatas, 1993; Fredholm et al., 1994; Watson, Gildelstone, 1994). The role of P_{2X}-purinoceptors possessing properties of intrinsic ion channels (Evans, 1996) in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport seems to be unlikely because of the electroneutral mode of operation of this carrier (Haas, 1994) and the lack of significant effect of ATP on intracellular content of monovalent ions (Table 4). This conclusion is also supported by data on the same level of inhibition of Na^+,K^+,Cl^- cotransport by ATP in ouabain-treated and monovalent ion-depleted MDCK cells (Fig. 7) as well as in shrunken and swollen cells (Fig. 8).

Both P_{2Y} and P_{2U} receptors coupled to heterotrimeric GTP-binding proteins (Fredholm et al., 1994) have been characterized pharmacologically in MDCK cells, but their relative contribution in the regulation of cellular function is still a matter of controversy (Firestein et al., 1996; Insel et al., 1996). It has been shown that inhibition of Gi in MDCK cells with pertussis toxin prevents ATP-induced $InsP_3$ production (Paulmichl et al., 1991; Yang et al., 1997). In contrast to this observation, we did not discern any effect of pertussis toxin on the regulation of Na^+,K^+,Cl^- cotransport by ATP (Table 1). In the majority of cells studied so far, both α-subunit of Gq and βγ-dimer derived from Gi activate PLCβ (van Biesen et al., 1996; Rocca et al., 1997). Our results demonstrate that an inhibitor of PLC, U73122 blocked ATP-induced $InsP_3$ production but did not affect Na^+,K^+,Cl^- cotransport (Fig. 1). Several laboratories have reported that ATP-triggers an elevation of $[Ca^{2+}]_i$ in MDCK cells due to $InsP_3$-induced Ca^{2+} release from intracellular store and influx via Ca^{2+}-release activated channels (CRAC) (Paulmichl et al., 1991; Delles et al., 1995). The present study confirms this observation (Fig. 2). However, neither basal Na^+,K^+,Cl^- cotransport nor its inhibition by ATP was affected under modulation of $[Ca^{2+}]_i$ homeostasis with thapsigargin, BAPTA and A23187 (Table 2). These results show that PLC activation triggered by P_{2Y}/P_{2U}-purinoceptors and coupled to Gq/Gi-mediated polyphosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ (Fig. 9) is not involved in the inhibition of Na^+,K^+,Cl^- cotransport by ATP.

Recently we reported that side by side with purinergic inhibition of Na^+,K^+,Cl^- cotransport, an activation of PKC with PMA leads to complete inhibition of this carrier (Gagnon et al., 1998). The present study confirms this observation. To investigate whether or not PKC is involved in purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport, we examined the effect of a selective inhibitor of PKC, calphostin C, on the regulation of the carrier by ATP using PMA-treated cells as a positive control. This compound as well as the nonselective inhibitor of PKC, staurosporine, abolished the inhibitory effect of PMA but did not modify the regulation of Na^+,K^+,Cl^- cotransport by ATP (Table 3). These results are in accordance with our previous data on ATP-induced inhibition of this carrier revealed in MDCK cells subjected to downregulation of PKC under long-term treatment with PMA (Gagnon et al., 1998). Depletion of MDCK cells with monovalent ions drastically diminished the inhibitory effect of PMA but did not alter the regulation of Na^+,K^+,Cl^- cotransport by ATP (Fig. 7). From this observation it seems that P_2-receptors are involved in regulating the number of functioning carrier or its turnover number, whereas PMA also increases the sensitivity of Na^+,K^+,Cl^- cotransport to inhibition by Cl^-_i or/and Na^+_i. Taken together, these results strongly suggest that the mechanism of the inhibition of Na^+,K^+,Cl^- cotransporter by PMA and ATP is different, and PMA/calphostin C-sensitive isoforms of PKC are not involved in the regulation of this carrier by ATP-coupled receptors. It should be mentioned, however, that apart from classic (PMA/diacylglycerol + Ca^{2+})-sensitive cPKC (PKC-α, βI, βII and γ) and novel PMA/diacylglycerol-sensitive Ca^{2+}-independent nPKC (PKC-δ, ε, η and ξ), the atypical (PMA/diacylglycerol + Ca^{2+})-insensitive aPKC (PKC-ζ, ι, λ, and μ), which are more resistant to staurosporine and other available PKC inhibi-

Fig. 9. Purinergic signaling in MDCK cells, AC—adenylate cyclase; CT—cholera toxin; DG—diacylglycerol; PL—phospholipids; PR—PGE_2 receptors; PT—pertussis toxin; RTK—receptor tyrosine kinases; TG—thapsigargin; ?—unidentified steps. → and — or - - - - activatory and inhibitory stimulus, respectively. Compounds used in the present study to modulate the activity of signaling pathways are shown in italics. For more details see text.

tors, have been characterized (Kikawa, Kishimoto, Nishizuka, 1989; Selbie et al., 1993; Akimoto et al., 1994). Considering this, the role of aPKC isoforms in inhibition of Na^+,K^+,Cl^- cotransport by ATP should be examined in additional experiments.

During the last few years, it has been documented that besides receptor tyrosine kinases, Gp-coupled receptors, including receptors of lysophosphatidic acid, $PGF_{2\alpha}$, α-thrombin, A_1-adenosine, angiotensin II, α_{1B}- and α_{2A}-adrenergic and M_1-cholinergic receptors, are able to activate MAP kinase. Both Ras-dependent and Ras-independent pathways of MAPK activation triggered by α-subunits and βγ-dimer of Gp have been identified in this signaling mechanism. The $p21^{ras}$-dependent pathway involves PLC-stimulated polyphosphoinositide hydrolysis, $InsP_3$-induced Ca^{2+}_i release, activation of Pyk2 and Src tyrosine kinase followed by recruitment of the adapter protein Shc and Crb2-Soc complex. The $p21^{ras}$-independent pathway is mediated by activation of diacylglycerol-dependent isoforms of PKC (Fig. 9) (for review see (van Biesen et al., 1996; Rocca et al., 1997; Force & Bonventre, 1988). Recently, it has been shown that purinergic activation leads to phosphorylation of MAPK in MDCK cells (Xing et al., 1997). Our results confirm this observation (Fig. 4). MAPK phosphorylation was also observed under treatment of MDCK cells with PMA (Figs. 3 and 5), suggesting that activation of the MAPK signaling cascade can be releated to ATP- and PMA-induced inhibition of Na^+,K^+,Cl^- cotransporter. However, the data presented below contradict this hypothesis.

1. Inhibition of MAPK with PD98059 did not prevent the inhibition of Na^+,K^+,Cl^- cotransport by ATP (Fig. 4).
2. Activation of MAPK with calf serum, EGF and thapsigargin (Figs. 3*A,* and *B* and 5) was not accompanied by inhibition of Na^+,K^+,Cl^- cotransport (Fig. 3*C,* Table 2 and our recent data (Gagnon et al., 1998).
3. Activation of MAPK signaling cascade by ATP was diminished after downregulation of PKC by long-term preincubation with PMA and after loading of MDCK cells with the Ca^{2+}_i-chelator, BAPTA (Fig. 5). This suggests that both $p21^{ras}$-dependent and $p21^{ras}$-insensitive PKC-dependent pathways are involved in purinergic-induced activation of MAPK (Fig. 9). On contrary, neither downregulation of PKC with PMA (Gagnon et al., 1998) nor modulation of Ca^{2+}_i with BAPTA (Table 2) affected the purinergic regulation of Na^+,K^+,Cl^- cotransporter.

Considering this, it may be concluded that activation of MAPK signaling is not involved in ATP-induced inhibition of Na^+,K^+,Cl^- cotransporter.

In MDCK cells, activation of P_2-receptors leads to increased PLA_2 activity via pertussis toxin-sensitive PKC- and MAPK-dependent signaling pathway. Indeed, it was shown that ATP-induced PLA_2 activation in these cells was accompanied by a massive release of arachidonic acid and PGE_2 as well as by cAMP production triggered by PGE_2 binding to Gs-coupled receptors (Insel et al., 1996; Post, Jacobsen & Insel, 1996; Firestein et al., 1996). ATP-induced arachidonic acid release can be completely blocked by down regulation of PKC and by addition of MAPKK inhibitor, PD98059 (Xing et al., 1997). As mentioned above, we did not reveal any effect of PD98059 (Fig. 4) and downregulation of PKC (Gagnon et al., 1998) on the regulation of Na^+,K^+,Cl^- cotransport by ATP. The inhibitory effect of ATP on this carrier also persisted after treatment of MDCK cells with cholera toxin (Table 1), exogenous arachidonic acid and inhibitors of PLA_2, cycloxygenase and lypoxygenase. The role of PGE_2-induced cAMP production in the regulation of Na^+,K^+,Cl^- cotransport seems to be unlikely because of the slight augmentation rather than inhibition of its activity observed in forskolin- (Gagnon et al., 1998) and cholera-toxin-treated cells (Table 1) and because of the negative results obtained with an inhibitor of PKA, H-89 (Table 3). Taken together, these results do not support the involvement of PLA_2 in ATP-induced inhibition of Na^+,K^+,Cl^- cotransport.

In conclusion, our results show that purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport in MDCK cells is not mediated by cholera and pertussis toxin-sensitive Gp. Inhibition of this carrier is independent of all known intracellular signaling pathways triggered by $P_{2U/2Y/2X}$-receptors, i.e., elevation of intracellular Ca^{2+} concentration, activation of PLC and PLA_2, diacylglycerol-sensitive isoforms of PKC, phosphorylation of MAPK, modulation of intracellular concentration of monovalent ions and cell volume. Taken together, these data suggest that ATP-induced inhibition of Na^+,K^+,Cl^- cotransport is triggered by a novel signal transduction pathway, not yet described in MDCK cells. It is well documented that the activity of ion channels can be regulated by a membrane-delimited pathway via direct interaction of channels with Gp-derived subunits (Brown & Birnbaumer, 1990). This pathway has recently been shown to be also involved in the regulation of Na^+/H^+ exchanger by G_{12} and G_{13} (Voyno-Yasenetskaya et al., 1994; Lin et al., 1996). The role of Gp distinct from Gs and Gi in purinergic-induced inhibition of Na^+,K^+,Cl^- cotransport deserves further study.

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