J. Membrane Biol. 186, 131–143 (2002)
DOI: 10.1007/s00232-001-0141-y

The Journal of
Membrane
Biology

Purinergic-Induced Ion Current in Monolayers of C7-MDCK Cells: Role of Basolateral and Apical Ion Transporters

N. Bourcier, R. Grygorczyk, M. Gekle*, Y. Berthiaume, S.N. Orlov
Centre de recherche, Centre hospitalier de l'Université de Montréal–Hôtel-Dieu, 3850 rue St-Urbain, Montréal, Quebec H2W 1T8, Canada
*Department of Physiology, University of Würzburg, Germany

Received: 10 October 2001/Revised: 14 December 2001

Abstract. This study examines purinergic modulation of short-circuit current (I_{SC}) in monolayers of C7- and C11-MDCK cells resembling principal and intercalated cells from collecting ducts. In C7 monolayers, basolateral and apical application of ATP led to similar elevation of I_{SC}, consisting of a transient phase with maximal I_{SC} increment of ~10 μA/cm^2 terminating in 2–3 min, and a sustained phase with maximal I_{SC} less than 2 μA/cm^2 and terminating in 10 min. ATP-induced I_{SC} was insensitive to the presence of Na^+, Cl^- and inhibitors of K^+ (Ba^{2+}, charibdotoxin (ChTX), clotrimazole (CLT), apamin) and Na^+ (amiloride) channels in the mucosal solution. Inhibitors of Cl^- channels, DIDS and NPPB, added to apical membranes at a concentration of 100 μM, did not affect ATP-induced I_{SC}, whereas at 500 μM, NPPB inhibited it by 70–80%. Substitution of Cl^- with NO_3^- in serosal medium decreased ATP-induced I_{SC} by 2–3-fold and elevation of $[K^+]_o$ from 6 to 60 mM changed its direction. Basolateral NPPB inhibited I_{SC} by 10-fold with ED_{50} of ~30 μM, whereas ChTX (50 nM) and CLT (2 μM) diminished this parameter by 30–50%. Suppression of Na^+, K^+, Cl^- cotransport with bumetanide did not affect the transient phase of ATP-induced I_{SC} and slightly diminished its sustained phase. ATP increased ouabain- and bumetanide-resistant K^+ (^{86}Rb) influx across the basolateral membrane by 7–8-fold, which was partially inhibited with ChTX and CLT. ATP-treated C11 cells exhibited negligible I_{SC}, and their presence did not affect I_{SC} triggered by ATP in C7 cells. Thus, our results show that transcellular ion current in ATP-treated C7 cells is mainly caused by the coupled function of apical and basolateral anion transporters providing transient Cl^- secretion. These transporters possess different sensitivities to anion channel blocker NPBB and are under the control of basolateral K^+ channels(s) inhibited by ChTX and CLT.

Key words: Principal and intercalated cells — Purinoceptors — Cl^- and K^+ channels — Na^+, K^+, Cl^- cotransport

Introduction

Renal epithelial cells from the collecting duct are major targets for the regulation of reabsorption of salt and osmotically-obliged water and acid-base homeostasis by hormones and neurotransmitters (Kirk, 1988; Burnatowska-Hledin & Spielman, 1989; Vander, 1991; Breyer & Ando, 1994; Giebisch, 1998). Apart from receptors of the "classic" regulators, such as vasopressin, bradykinin, atrial natriuretic peptide, prostanoids, mineralocorticoids and catecholamines, epithelial cells from collecting ducts express ATP- and UTP-sensitive P_{2Y}-purinoceptors (Bailey, Hillman & Unwin, 2000; Chan, Unwin & Burnstock, 2001, Cuffe et al., 2000).

Data on the involvement of P_{2Y}-purinoceptors in the regulation of transepithelial ion fluxes are mainly obtained in cells derived from distal tubules of the Madin-Darby canine kidney (MDCK)[1] (Simmons,

Correspondence to: S.N. Orlov; email: sergei.n.orlov@umontreal.ca

[1] *Abbreviations used:* C7 and C11—cell lines derived from MDCK cells and resembling principal and intercalated cells, respectively; CFTR—cystic fibrosis transmembrane regulator; ChTX—charybdotoxin; CLT—clotrimazole; DIDS—4,4′-diidothiocyanostilbene-2,2′-disulfonic acid; EIPA—ethylisopropyl amiloride; I_{SC}—short-circuit current; $I_{SC\text{-}MAX\text{-}TR}$ and $I_{SC\text{-}MAX\text{-}SS}$—the maximal values of I_{SC} during transient and sustained phases, respectively; MDCK—Madin Darby canine kidney; NKCC—Na^+, K^+, Cl^- cotransport; NPPB—5-nitro-2-(3-phenylpropylamino)benzoic acid; Q_T and Q_{SS}—total charge movement and charge movement during sustained phase, respectively.

1981a,b; Zegarra-Moran, Rommeo & Galietta, 1995; Woo et al., 1998). It should be underlined, however, that similarly to the distal epithelium, commercially-available stocks of MDCK cells are heterogeneous with different proportions of principal and intercalated cells from the collecting duct (Barker & Simmons, 1981; Simmons, 1990). Recently, using a low-density seeding strategy these cells were separated and called C7-MDCK and C11-MDCK, respectively (Gekle et al., 1994). With these cells, we observed drastic differences in the baseline activity of monovalent ion transporters and their modulation by P_{2Y}-puronoceptors (Orlov et al., 1999). We also demonstrated that purinergic inhibition of Na^+, K^+, Cl^- cotransport (NKCC) and activation of MAP kinase, recently discovered in MDCK cells (Xing et al., 1997; Gagnon et al., 1998, 1999), is limited to C11 cells (Orlov et al., 1999). Considering these data, we designed this study to characterize purinergic-induced transcellular ion current in monolayers of C7 and C11 cells and to identify apical and basolateral ion transporters involved in this phenomenon. Epinephrine, an adrenoceptor agonist, is known to be another potent activator of transcellular ion current in monolayers of MDCK cells (Brown & Simmons, 1981; Simmons, Brown & Rugg, 1984; Brown, Rugg & Simmons, 1986; Simmons, 1991b; 1992). Hence, we used this compound to examine the specificity of P_{2Y}-purinoceptor signaling.

Materials and Methods

Cell Culture

C7 and C11 cells were obtained from commercially-available MDCK stocks, as described previously in detail (Gekle et al., 1994). The cells were cultured in DMEM 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. Upon reaching subconfluency, they were passaged by treatment in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline with 0.1% trypsin and scraped from the flasks with a rubber policeman. Dispersed cells were counted and inoculated at 1.25×10^3 cell/cm^2 in 24-well plates or in 1-cm^2 permeable inserts (Corning Brand transwell plate inserts, Fisher, Montreal, Canada). Corning Brand transwell clear inserts were used for phase contrast microscopy.

Electrical Measurements

After 3–4 days of seeding on transwell inserts, C7 and C11 cells developed transepithelial resistance (R_{te}) that was 20–40- and 3–5-fold higher compared with empty inserts. R_{te} values measured with EVOM™ (World Precision Instruments, Sarasota, FL) were stable during the next 1–2 days. For short-circuit current (I_{SC}) measurements, the transwell inserts containing monolayers with R_{te} in the range from 1,500 to 3,500 $\Omega \times cm^2$ for C7 cells and from 250 to 350 $\Omega \times cm^2$ for C11 cells were mounted between halves of an Ussing chamber (Warner Instrument Corp., Hamden, CT). Fluid in each half of the chamber was connected via KCl-agar bridges to voltage and current electrodes and clamped at 0 mV using an EC-825 epithelial voltage clamp amplifier (Warner Instrument Corp.). Basolateral and apical solutions, 16 and 14 ml, respectively, containing 2% calf serum and HEPES-$NaHCO_3$-buffered Dulbecco's modified Eagle medium (DMEM, pH 7.4) were circulated by air-lifting with 5% CO_2/95% air and kept at 37°C by water jacket.

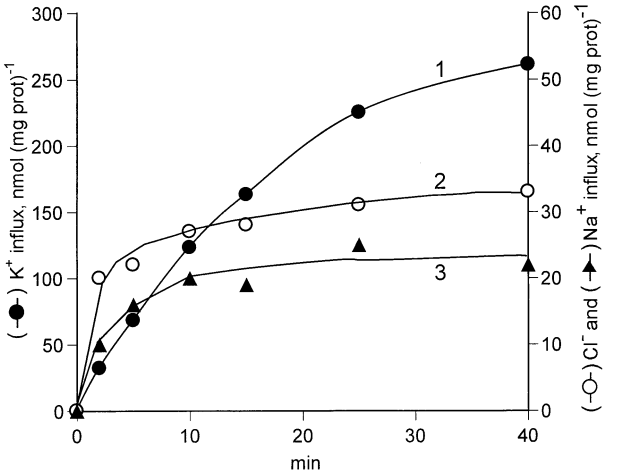


Fig. 1. Kinetics of a ^{86}Rb (*1*) ^{36}Cl (*2*) and ^{22}Na (*3*) uptake by C7 cells. The cells were preincubated for 30 min in medium B. Then, this medium was aspirated, and medium B containing ^{86}Rb, ^{36}Cl and ^{22}Na was added. For more details, *see* Methods. Means from experiments performed in triplicate are shown.

Ion Fluxes in Cells Grown on Impermeable Support

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 additions indicated in the table and figure legends. 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 by 0.25 ml of the same medium containing 0.5–1 μCi/ml $^{86}RbCl$ or 2–4 μCi/ml $^{22}NaCl$ and $H^{36}Cl$. Isotope 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, and ion uptake was calculated as $V = A/am$ where A is the radioactivity in the sample (cpm), a is the specific radioactivity of ^{86}Rb (K^+), ^{36}Cl or ^{22}Na (cpm/nmol) in the incubation medium and m is the protein content in the sample (mg).

Figure 1 shows that the kinetics of ^{86}Rb uptake by C7 cells in the absence of any ion transport inhibitors were linear up to 15 min. Considering these results, in further experiments we limited incubation time with ^{86}Rb to 10–15 min. In contrast to ^{86}Rb, half-maximal uptake of ^{36}Cl and ^{22}Na was observed in 2 min (Fig. 1), which complicates the application of this technique for analysis of the relative contribution of ion transporters in inwardly-directed Cl^- and Na^+ fluxes.

The presence of ouabain did not affect the absolute values of bumetanide-sensitive K^+ influx in C7 cells (Table 1). Based on this observation we studied the activity of Na^+, K^+, Cl^- cotransport in the presence of ouabain that allowed us to increase an accuracy of the measurement.

Table 1. Effect of ouabain and bumetanide on K^+ influx in C7-MDCK cells

	Additions, μM	K^+ influx, nmol/mg prot/15 min
1.	None (control)	321 ± 39
2.	Ouabain, 100	122 ± 16
3.	Ouabain-sensitive component $\Delta_{1,2}$	199
4.	Ouabain, 100 + bumetanide, 10	14 ± 2
5.	Ouabain-resistant bumetanide-sensitive component, $\Delta_{2,4}$	108
6.	Bumetanide, 10	203 ± 17
7.	Bumetanide-Sensitive $\Delta_{1,6}$	118

Cells seeded in 24-well plates were preincubated 30 min in medium B. Then, medium was aspirated and 0.25 ml of medium B with compounds listed in the left column was added. In 5 min 0.25 ml of medium B containing 1 μCi/ml ^{86}Rb was added and isotope uptake was terminated in 15 min. The values of final concentrations of ouabain and bumetanide are indicated. Means ± SE from experiments performed with quadruplicate are shown.

K^+ Influx Across Basal and Apical Membranes in Cell Monolayers

To analyze the effect of ATP on K^+ influx across basolateral and apical membranes, we used C7 monolayers formed on permeable transwell inserts. The cell monolayers were preincubated for 30 min at 37°C in DMEM containing 2% calf serum. Then, the inserts were transferred to plates containing 1.5 ml of prewarmed DMEM with the same addition of serum, and 0.5 ml of the same medium was added on the apical surface of the monolayers. The serosal or mucosal medium also contained ~0.5 μCi/ml ^{86}Rb, ATP, epinephrine and the ion transport inhibitors indicated in the figure and table legends. To terminate isotope uptake, the plates were transferred to an ice-cold water bath, washed 4 times in 50 ml of ice-cold medium C, solubilized with SDS/EDTA mixture, and the radioactivity of the cell lysate was counted. To measure transepithelial ion fluxes, serosal or mucosal solutions were collected and their radioactivity was counted. The values of ion fluxes were calculated as described above and normalized per monolayer area. In preliminary experiments, we observed that the kinetics of ^{86}Rb accumulation within monolayers from the serosal and mucosal media were linear up to 20 min of incubation. Based on these results, we limited the incubation time to 10 min in further experiments.

Exchangeable K^+, Cl^- and Na^+

Cells seeded on transwell inserts were incubated for 2 hr in DMEM containing 2% calf serum, ~0.5 μCi/ml ^{86}Rb and 2–3 μCi/ml ^{22}Na or ^{36}Cl added on both sides of the monolayer. Then, the mucosal solution was aspirated, and the inserts were washed 4 times in 50 ml of ice-cold medium C and solubilized with SDS/EDTA mixture. The content of intracellular exchangeable K^+, Na^+ and Cl^- was calculated as described above and normalized per monolayer area.

Chemicals

DMEM, calf serum and other ingredients for cell cultures were obtained from Gibco Laboratories (Burlington, Canada). Ouabain, bumetanide, amiloride, DIDS, EIPA, apamin, charybdotoxin, clotrimazole, NPPB and trypsin were from Sigma-RBI (St. Louis, MO). Radiochemicals were provided by Dupont (Boston, MA). Salts and buffers were obtained from Sigma and Anachemia Science (Montreal, Canada).

Results

Comparison of ATP- and Epinephrine-induced I_{SC} in C7 Cells

Baseline values of I_{SC} in C7 monolayers varied from ~1 to 3 μA/cm^2, which is in accordance with previously reported data (Blazer-Yost, Record & Oberleithner, 1996; Lahr et al., 2000). Figure 2*A* shows a typical record of I_{SC} modulation by serosal application of ATP and epinephrine. Positive deflection of I_{SC} triggered by these compounds indicates cation movement from apical to serosal medium or/and anion movement in the opposite direction. In both cases, the kinetics of I_{SC} modulation were composed of the rapid initial phase followed by the sustained phase seen up to 5–10 min in ATP-treated cells and for 20 min and more in cells treated with epinephrine. Neither apical nor basolateral application of 10 μM adenosine affected I_{SC} (*data not shown*), indicating lack of the contribution of ecto-ATPase/P_1-receptor-coupled pathway in ATP-induced signaling.

To analyze the relative contribution of the rapid and sustained phases to the overall signal, we compared the values of the maximal I_{SC} increment during the transient and sustained phases ($I_{SC\text{-}MAX\text{-}TR}$ and $I_{SC\text{-}MAX\text{-}SS}$, respectively), total charge movement for 10 min (600 sec) calculated as $Q_T = \int_0^{600} I_{SC}dt$, and long-lasting charge movement during the sustained phase measured between 2 and 10 min and calculated as $Q_{SS} = \int_{120}^{600} I_{SC}dt$ (Fig. 2*A*). This analysis showed that: (i) In contrast to ATP, apical application of epinephrine did not affect I_{SC} (Fig. 2*B* and *C*), which is in accordance with data obtained on MDCK cells (Brown & Simmons, 1981). (ii) I_{SC} parameters triggered by basolateral and apical ATP were about the same. (iii) The sustained phase contributed ~35% and 75% of the total charge transfer in cells treated with ATP and epinephrine, respectively. In further experiments, we used $I_{SC\text{-}MAX\text{-}TR}$ and Q_{SS} values to study purinergic modulation of the transient and sustained phases.

Effect of Ion Transport Inhibitors on ATP-induced I_{SC}

Because of the similarity of kinetics of I_{SC} modulation by apical and basal application of ATP (Fig. 2), this compound was added to the basolateral surface of the monolayer in initial experiments. The ion transport inhibitors affecting I_{SC} triggered by basal ATP were further examined in cells treated with apical ATP.

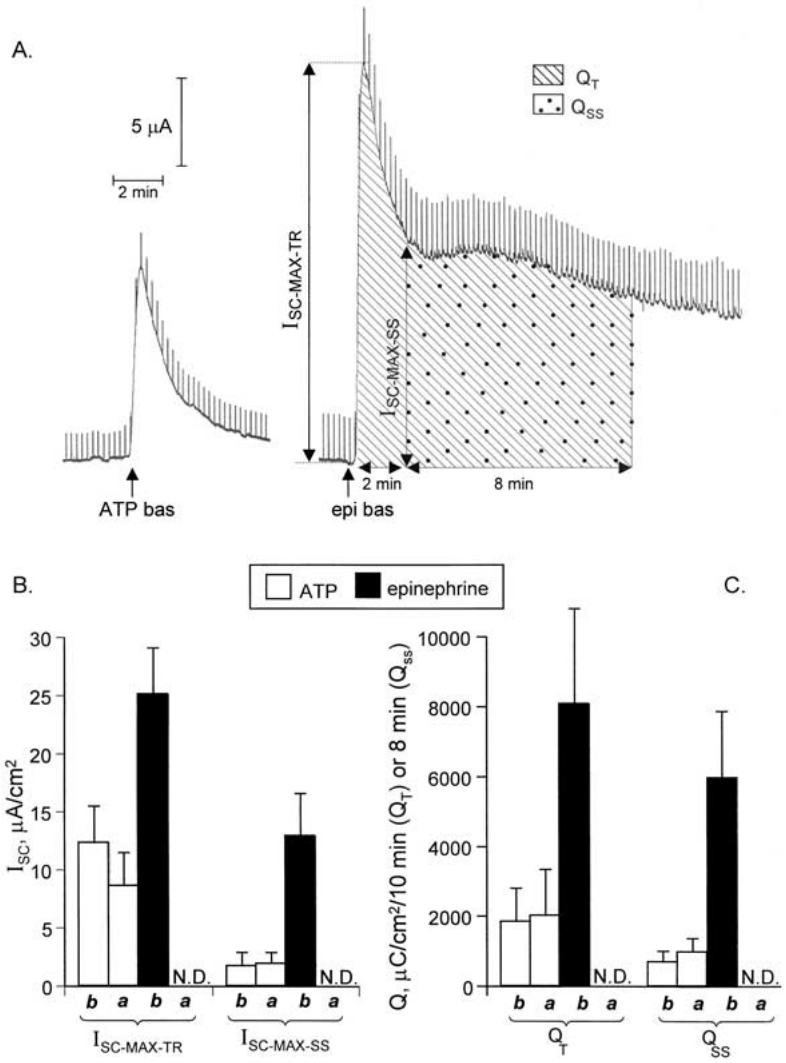


Fig. 2. ATP- and epinephrine-induced I_{SC} in C7 monolayers. *Top*. (*A*) Typical records of the kinetics of I_{SC} modulation by basal application of 100 μM ATP and 1 μM epinephrine (*epi*). *Bottom*. Effect of basal (*b*) and apical (*a*) application of ATP and epinephrine on the maximal increment of I_{SC} during transient and sustained phases ($I_{SC\text{-}MAX\text{-}TR}$ and $I_{SC\text{-}MAX\text{-}SS}$, respectively) (*B*), and on total transcellular charge movement (Q_T) and charge movement during the sustained phase (Q_{SS}). (*C*) Means ± SE obtained from 12 experiments are shown. *N.D.*: these values were below the limit of detection.

Epinephrine-treated cells were used to reveal purinoceptor-specific intermediates of ion flux regulation.

Table 2 shows that basal application of $BaCl_2$, an inhibitor of diverse K^+ channels, as well as charybdotoxin (ChTX) and clotrimazole (CLT), potent inhibitors of Ca^{2+}-activated K^+ channels with high (BK_{Ca}) and intermediate (IK_{Ca}) conductance, decreased ATP-triggered $I_{SC\text{-}MAX\text{-}TR}$ and Q_{SS} by 30–50%. None of these compounds added to the apical surface significantly affected ATP-induced I_{SC}. The addition of bumetanide, an inhibitor of NKCC, to basolateral membranes did not alter $I_{SC\text{-}MAX\text{-}TR}$ and attenuated Q_{SS} by ~40%. ATP-induced transcellular current was not affected by inhibitors of the Na^+, K^+ pump (100 μM ouabain), mini-K_{Ca} channels (1 μM apamin), epithelial Na^+ channels (10 μM amiloride) and Na^+/H^+ exchanger (1 μM EIPA) applied to both surfaces of the monolayer (*data not shown*). The negative results with amiloride are consistent with data obtained under analysis of ATP-induced I_{SC} in M-1 mouse cortical collecting duct cells (Cuffe et al., 2000).

NPPB and DIDS were employed to study the role of Cl^- channels in ATP-triggered I_{SC}. Neither basal nor apical addition of 100 μM DIDS affected ATP-induced I_{SC} (*data not shown*). The addition of 100 μM NPPB to the basolateral surface of the monolayer led to 10-fold attenuation of ATP-induced $I_{SC\text{-}MAX}$, whereas its apical application decreased this parameter and Q_{SS} by 20–25% and 30–40%, respectively (Table 2). To further examine the role of apical and basolateral anion channels, we compared dose dependencies of the NPPB effect at concentrations up to 500 μM. Because NPBB also affects NKCC (*see* below), this experiment was carried out in the presence of bumetanide. Figure 3 shows that NPBB applied to the basolateral and apical membranes inhibited I_{SC} with ED_{50} of ~30 and 400 μM, respectively.

We did not observe any difference in the effect of the compounds listed in Table 2 as modulators of I_{SC} triggered by apical or basal ATP. In contrast to ATP, basolateral application of inhibitors of K^+ channel blockers and 100 μM NPPB did not affect the maxi-

Table 2. Effect of ion transport inhibitors on I_{SC} in C7-MDCK cells triggered by ATP and epinephrine (epi)

Additions	Concentration μM	Side added	$I_{SC\text{-}MAX}$, %			Q_{SS}, %		
			ATP		epi	ATP		epi
			basal	apical	basal	basal	apical	basal
Bumetanide	10	basal	99 ± 11	83 ± 12	98 ± 11	**62 ± 23**	70 ± 12	**32 ± 12**
Bumetanide	10	apical	104 ± 7	—	—	95 ± 6	—	—
$BaCl_2$	2000	basal	**61 ± 7**	**58 + 11**	91 ± 6	**45 ± 7**	**49 ± 18**	**57 ± 9**
$BaCl_2$	2000	apical	98 ± 10	—	—	90 + 8	—	—
ChTX	0.05	basal	**71 ± 8**	**70 ± 9**	87 + 12	**65 ± 7**	**63 ± 18**	87 ± 14
ChTX	0.05	apical	103 ± 12	—	—	100 ± 6	—	—
Clotrimazole	2	basal	**75 ± 6**	**70 ± 8**	85 ± 9	**70 ± 5**	**60 ± 7**	**72 ± 12**
Clotrimazole	2	apical	107 ± 11	—	—	—	—	—
NPPB	100	basal	**10 ± 8**	**12 ± 6**	103 ± 11	8 ± 4	**8 ± 5**	**64 ± 6**
NPPB	100	apical	76 ± 26	82 ± 6	89 ± 8	**68 ± 15**	**52 ± 17**	**29 ± 16**

Bumetanide, $BaCl_2$, ChTX, clotrimazole and NPPB were added 1-2 min before ATP (100 μM) or epinephrine (epi, 1μM). The $I_{SC\text{-}MAX}$ and Q_{SS} values in cells treated with ATP or epinephrine in the absence of inhibitors were taken as 100%. Means ± SE from 6 (ATP-basal) or 4 (ATP-apical and epinephrine-basal) experiments are given. Differences with $p < 0.05$ compared to controls are shown in bold. — indicates that these values were not determined.

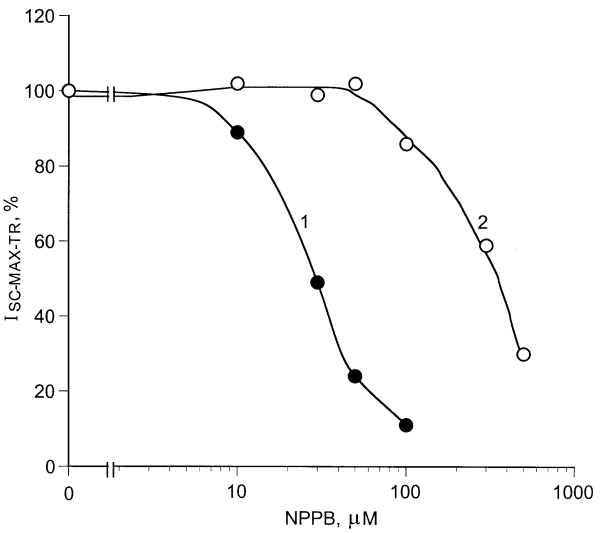


Fig. 3. Dose-dependency of the effect of basolateral (*curve 1*) and apical (*curve 2*) application of NPPB on the maximal increment of ATP-induced I_{SC} in C7 monolayers. I_{SC} was triggered by 100 μM ATP added to the basolateral surface of the monolayer. NPPB was added 1–2 min before ATP. 10 μM bumetanide was added to the serosal medium simultaneously with ATP. The values of $I_{SC\text{-}MAX\text{-}TR}$ in the absence of NPPB were taken as 100%. Means from experiments performed in triplicate are shown.

mal values of epinephrine-induced I_{SC}, whereas the addition of 500 μM NPPB to the apical surface inhibited epinephrine-induced I_{SC} to about the same extent as observed with ATP-treated cells (Table 2).

Effect of Extracellular Monovalent Ions

Modulation of Na^+, Cl^- and K^+ content in mucosal medium did not affect I_{SC} parameters triggered by basolateral ATP (Fig. 4). Equimolar substitution of NaCl with N-methyl-D-glucamine chloride in serosal medium did not significantly alter ATP-induced $I_{SC\text{-}MAX\text{-}TR}$ but decreased Q_{SS} by 60–70%. Substitution of Cl^- with NO_3^- in serosal medium decreased ATP-induced $I_{SC\text{-}MAX\text{-}TR}$ and O_{SS} by 2–3- and 5-fold, respectively. We also observed that elevation of K^+ in serosal solution from 6 to 60 μM resulted in an opposite direction of ATP-induced I_{SC} (Fig. 4). The same effect of extracellular ions on I_{SC} was revealed in cells treated with apical ATP (*data not shown*).

Similarly to ATP, modulation of monovalent ion content in mucosal medium did not significantly affect epinephrine-triggered I_{SC} (*data not shown*). In contrast to ATP, epinephrine-induced I_{SC} was sharply diminished in the absence of Na^+ in the serosal medium and was absent in high-K^+ medium (Fig. 5).

Comparison of C7 and C11 Cells

In collecting ducts, 10% to 30% of the epithelial area is covered by intercalated cells (Schwartz, 1995). Do these cells contribute to purinergic regulation of transepithelial ion fluxes? To answer this question, we studied I_{SC} in C11 monolayers and in monolayers with different relative content of C7 and C11 cells. Phase contrast microscopy showed clear differences in the morphology of these cells and their clustering when they were seeded at 1:1 initial density (Fig. 6*A*). We did not observe any effect of ATP on I_{SC} in C11 monolayers (Fig. 6*C*, *c*). Elevation of the relative content of C11 cells in monolayers up to 50% decreased R_{te} from 1,837 ± 165 to 509 ± 51 $\Omega \times cm^2$, which was close to the R_{te} in monolayers formed by C11 cells alone (Fig. 6*B*, curve 1). In contrast to R_{te}, the maximal I_{SC} values triggered by ATP and epinephrine decreased proportionally to the C11/C7 ratio (Fig. 6*B*, curves *2* and *3*). The presence of C11-

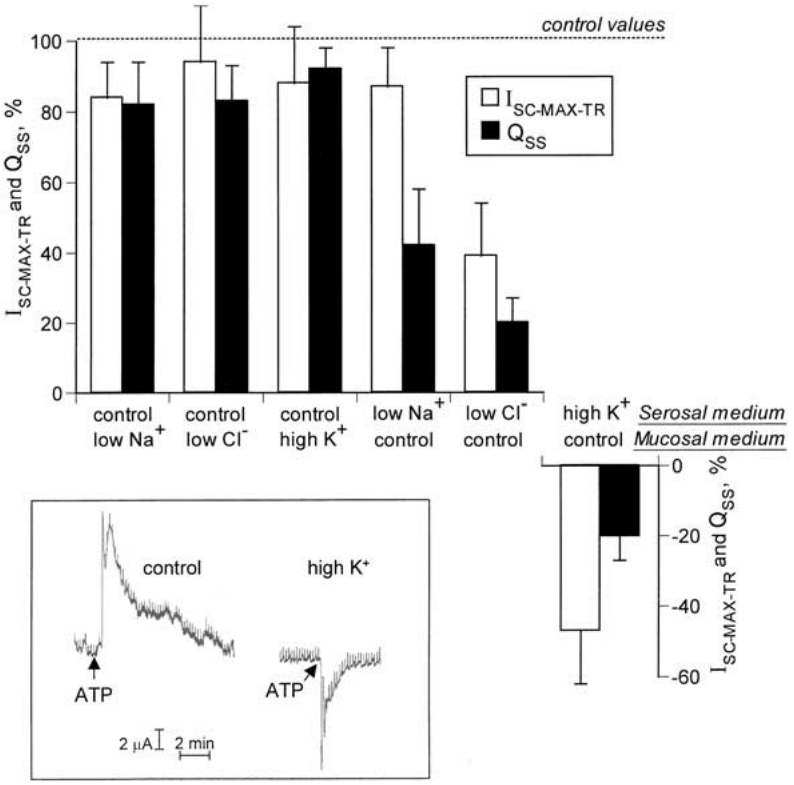


Fig. 4. Effect of extracellular Na^+, Cl^- and K^+ on I_{SC} triggered in C7 monolayers by basal application of ATP. The cells seeded on permeable inserts were preincubated for 10 min in physiologically balanced solution containing in mM: 130 NaCl, 5 KC1, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 20 HEPES-tris (pH 7.4), 10 glucose, 1 glutamine, 0.7 KH_2SO_4 and 1% calf serum (*control*) or in media with equimolar substitution of NaCl by N-methyl-D-glucamine chloride *(low Na^+ medium)*, equimolar substitution of NaCl and KCl by $NaNO_3$ and KNO_3, (*low Cl^- medium*), and in the presence of 75 mM NaCl and 60 mM KCl (*high K^+ medium*). Then, 100 μM ATP was added to the serosal solution and I_{SC} was registered for the next 10 min. $I_{SC-MAX-TR}$ and Q_{SS} values generated by ATP in the control medium were taken as 100% and are shown by the broken line. All media were bubbled with air. Means ± SE from 3 experiments are shown. *Insert:* Typical records of I_{SC} modulation by basal application of 100 μM ATP in control and in high-K^+ serosal medium.

MDCK cells did not affect the kinetics of I_{SC} triggered by ATP (Fig. 6*C*, *b*) and epinephrine (*data not shown*).

Effect of ATP on K^+ Influx in C7 Cells Grown on Impermeable Support

In randomly-polarized C7 cells seeded on regular plastic plates, bumetanide-sensitive and bumetanide-resistant K^+ (^{86}Rb) influx was increased for 10 min after simultaneous addition of ATP and isotope by ~30% and 100%, respectively (Fig. 7). Neither bumetanide-sensitive nor bumetanide-resistant K^+ influx was altered when ATP was added 30 min before ^{86}Rb (*data not shown*). The latter results are in accordance with previously-reported data (Orlov et al., 1999) and indicate the transient pattern of activation of K^+ fluxes by ATP. In contrast to ATP, epinephrine did not significantly affect K^+ influx in C7 cells (Fig. 7).

To identify the ion transporters involved in activation of (ouabain-bumetanide)-resistant K^+ influx by ATP, we studied the effect of K^+ and Cl^- channel inhibitors. Previously, we reported that 30-min preincubation with 5 mM $BaCl_2$ sharply attenuated the activity of NKCC in MDCK cells (Gagnon et al., 1999). Considering these results together with data on partial suppression of the sustained phase of ATP-induced I_{SC} by bumetanide (Table 2), the effect of the ion channel blockers on NKCC was also investigated. Figure 8 shows that inhibitors of K^+ channels attenuated bumetanide-resistant K^+ influx triggered by ATP (curve *1*) with ED_{50} of ~0.5 mM, 0.5 μM and 5 nM for $BaCl_2$, CLT and ChTX, respectively. However, it must also be mentioned that $BaCl_2$ and CLT dose-dependently inhibited NKCC with ED_{50} of ~2 mM and <2 μM, respectively (curve *2*). NPPB inhibited baseline NKCC and ATP-induced bumetanide-resistant K^+ influx with about the same efficiency (ED_{50} ~50 μM).

Unidirectional K^+ Fluxes in Monolayers

Several laboratories have reported that the polarization of epithelial cells controlled by their confluency and the presence of porous support sharply affect the functional activity of apical and basolateral ion transporters (Ponce, Contreras & Cereijido, 1991; Moyer et al., 1998; Mohamed et al., 1997). Considering this, we studied the baseline activity of K^+ transporters and their purinergic regulation in C7 monolayers. Table 3 shows that the rate of ^{86}Rb accumulation from serosal medium within C7 monolayers was diminished by ~2.5-fold in the presence of ouabain and was slightly inhibited (~15%) by bumetanide applied to the basolateral surface. ^{86}Rb uptake within monolayers from mucosal medium was 3–4-fold lower and, as predicted, was insensitive to the presence of ouabain and bumetanide. In contrast

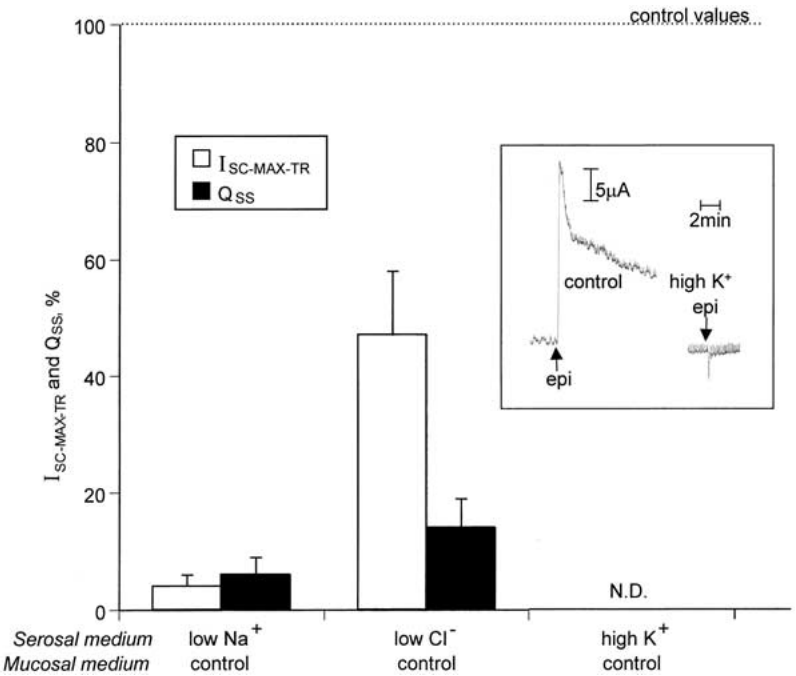


Fig. 5. Effect of extracellular Na^+, Cl^- and K^+ on I_{SC} triggered in C7 monolayers by basal application of epinephrine. The cells seeded on permeable inserts were preincubated for 10 min in physiologically balanced solution (*control*) or in media with altered concentration of Na^+, Cl^- and K^+. Then, 1 μM epinephrine was added to the serosal solution, and I_{SC} was registered for the next 10 min. $I_{SC-MAX-TR}$ and Q_{SS} values generated by epinephrine in the control medium were taken as 100% and are shown by broken line. *N.D.*: values could not be detected. Means ± SE from 3 experiments are shown. For composition of media, *see* Fig. 4 legend. *Insert:* Typical records of I_{SC} modulation by basal application of 1 μM epinephrine in control and in high-K^+ serosal medium.

to ^{86}Rb accumulation within the cells, the values of transepithelial basal-to-apical and apical-to-basal isotope fluxes varied in the range from 3 to 4 and from 4 to 6 nmol per cm^2 per 10 min, respectively (Table 3), thus indicating a slight K^+ secretion under baseline conditions.

Figure 9*A* shows that both basal and apical addition of ATP led to ~3-fold elevation in the rate of ^{86}Rb accumulation within monolayers from serosal solution, whereas epinephrine increased this parameter by 30–40% only. Drastic activation of ^{86}Rb influx within the cells in the presence of ATP was not accompanied by its accumulation in mucosal medium. Neither ATP nor epinephrine affected ^{86}Rb accumulation in monolayers when isotope was added to the apical surface (*data not shown*). We also did not observe any effect of 10-min incubation with ATP or epinephrine on the intracellular exchangeable K^+, Na^+ and Cl^- content (Table 4).

ATP-induced ^{86}Rb uptake from serosal medium was inhibited by 40–60% with Ba^{2+}, ChTX and CLT and by 20% with NPPB, but was insensitive to ouabain and bumetanide (Fig. 9*B*). In the presence of ouabain and bumetanide, ATP and epinephrine increased the rate of ^{86}Rb transport across basal membranes by 7–8-fold and by ~30%, respectively (Table 5).

Viewed collectively, our results show that the formation of cell monolayers on permeable support drastically affects both the baseline activity of ion transporters and their regulation by ATP. Indeed, NKCC activity measured as bumetanide-sensitive ^{86}Rb uptake contributed 43% and 1% to total K^+ influx in C7 cells grown on impermeable plastic and in C7 monolayers, respectively (Tables 1 and 3). In contrast, K^+ channels and other ion transporters providing (ouabain + bumentanide)-resistant fluxes contribute 4% and 35% to total K^+ influx in randomly-polarized cells and monolayers (Tables 1 and 3). In the presence of ouabain and bumetanide, ATP led to ~2- and 7–8-fold elevation of K^+ influx in cells grown on plastic (Fig. 8) and in cell monolayers (Table 5). In MDCK cells seeded on impermeable support, the ratio of intracellular exchangeable Na^+, Cl^- and K^+ content was close to 1:4:13 (Gagnon et al., 1999) whereas in C7 monolayers this ratio, measured with the same experimental approach, was 1:1.4:2 (Table 4). These observations disclose the limitations in usage of cells seeded on regular plastic for the study of epithelial cell function.

Discussion

Using MDCK, it was demonstrated earlier that long-lasting I_{SC} triggered by ATP is caused by Cl^- secretion mediated by basolateral NKCC and a non-identified apical Cl^- transporter (Simmons, 1981b). Later, it was shown that the effect of ATP on I_{SC} is mediated by P_{2Y}-purinoceptors (Zegarra-Moran et al., 1995). Neither ion transporters involved in the transient phase of P_{2Y}-purinoceptor-induced I_{SC} nor the relative contribution of principal and intercalated cells to overall I_{SC} has been investigated. In the present study, we used C7 and C11 cells, which are highly abundant with principal and intercalated cells, respectively (Gekle et al., 1994). Our results reveal that ATP-induced I_{SC} is negligible in C11 compared to C7 cells. We also observed that NKCC does not contribute to the transient elevation of

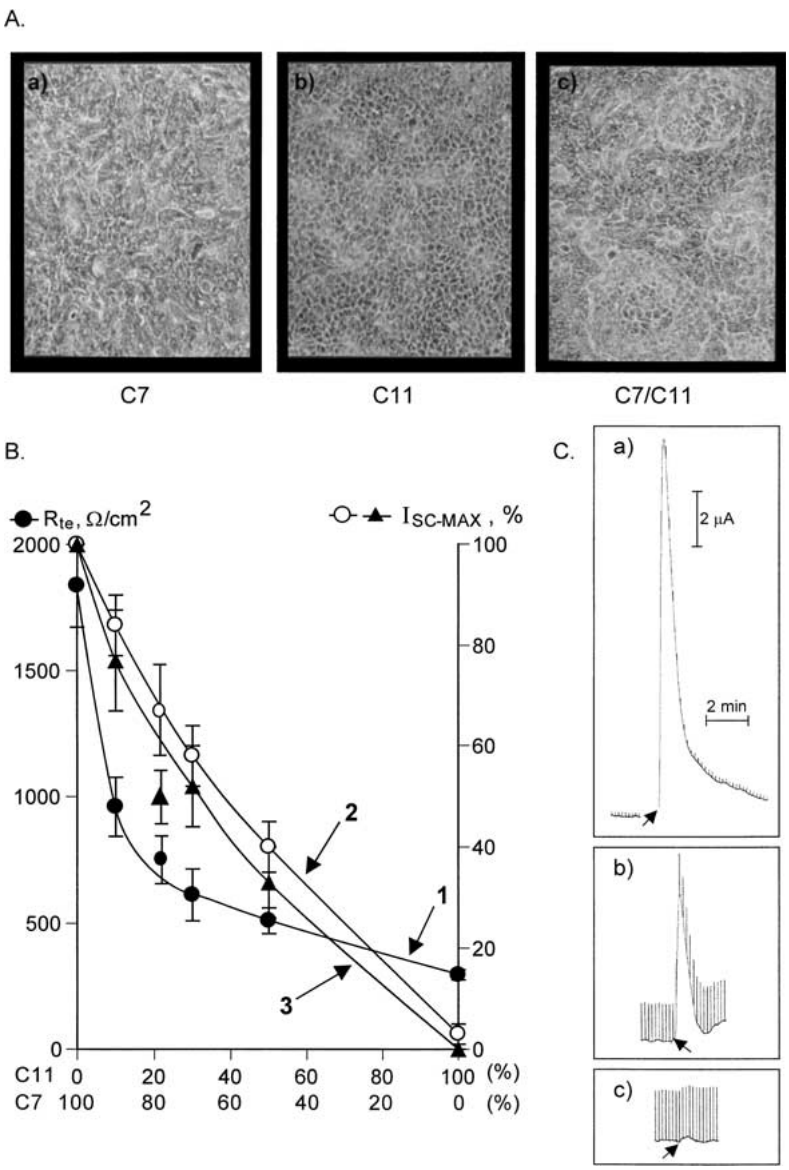


Fig. 6. (*A*) Phase contrast microscopy of monolayers formed by C7 (*a*), C11 (*b*) and C7 and C11 cells seeded at initial 1:1 density (*c*). (*B*) Dependence of transepithelial resistance (R_{te}, *curve 1*)) and maximal I_{SC} values triggered by ATP (*curve 2*) and epinephrine (*curve 3*) on the initial density of C7 and C11 cells. 100 μM ATP and 1 μM epinephrine were applied on the basolateral surface of the monolayer. The relative density of C7 and C11 cells seeded on transwell inserts 3 days before measurement are shown in the X axis. $I_{SC-MAX-TR}$ values triggered by ATP and epinephrine in monolayers of C7 cells were taken as 100%. Means ± SE from 3 experiments are shown. (*C*) Representative kinetics of I_{SC} triggered by ATP in C7 (*a*) and C11 (*c*) monolayers. *b*) C7- and C11-MDCK cells were seeded at 1:1 initial density.

I_{SC} in ATP-treated C7 cells and transcellular Cl^- movement is mainly mediated by the coupled function of apical and basolateral transporters possessing distinct sensitivity to NPPB. Our results also illustrate a key role of basolateral ChTX- and CLT-sensitive K^+ channels in the activation of Cl^- secretion. In addition, we observed that both baseline activity of monovalent ion transporters and their regulation by P_{2Y}-purinoceptors in C7 monolayers and randomly-polarized C7 cells grown on impermeable support are drastically different.

ATP-Induced Cl^- Secretion in C7 Cells: A Key Role of NPPB-sensitive Basolateral and Apical Transporters

We did not observe any effect of modulation of Na^+, K^+ and Cl^- concentration in mucosal medium on ATP-induced I_{SC} in C7 monolayers, whereas substitution of Cl^- with NO_3^- in serosal solution sharply attenuated transcellular ion current (Fig. 4). These results show that basal-to-apical Cl^- movement rather than apical-to-basal cation fluxes contributes to I_{SC} deflection in ATP-treated cells. This conclusion is also supported by negative results obtained in the study of the effect of inhibitors of K^+ channels (Ba^{2+}, ChTX, CLT, Table 2) and epithelial Na^+ channels (amiloride, *data not reported*) applied to the apical side of the monolayer.

Our results reveal that ATP-induced I_{SC} was resistant to DIDS and inhibited by basolateral and apical application of NPPB with ED_{50} of ~30 and 400 μM, respectively (Fig. 3). Both DIDS and NPPB are known to be potent inhibitors of several types of Cl^- channels. Thus, DIDS blocks large-conductance Cl^- channels, outwardly rectifying Cl^- channels and

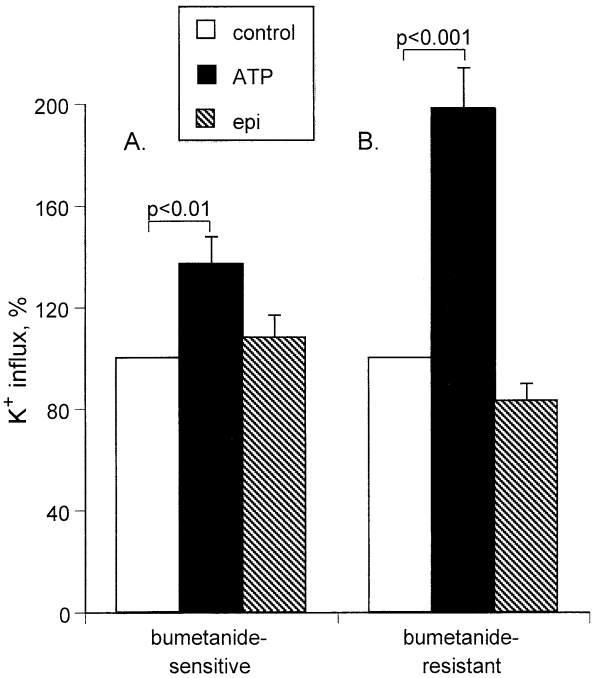


Fig. 7. Effect of ATP and epinephrine (*epi*) on bumetanide-sensitive (*A*) and bumetanide-resistant (*B*) K^+ influx in C7 cells. The cells seeded on regular plastic plates were preincubated for 30 min in 1 ml medium B. Then, the medium was aspirated, and 0.25 ml of medium B containing 0.5 μCi/ml ^{86}Rb, 100 μM ouabain with or without 10 μM bumetanide, 100 μM ATP and 1 μM epinephrine was added. Isotope uptake was terminated in 10 min. The values of bumetanide-sensitive and bumetanide-resistant components of K^+ influx in the absence of ATP and epinephrine were taken as 100%. For the absolute values *see* Table 1. Means ± SE from 5 experiments performed in quadruplicate are shown.

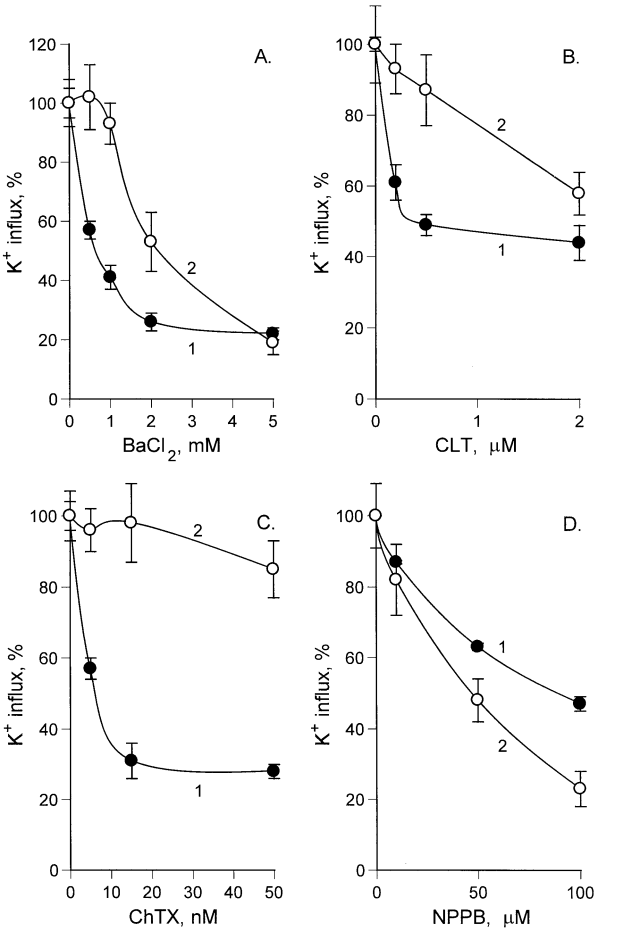


Fig. 8. Effect of $BaCl_2$ (*A*), CLT (*B*), ChTX (*C*) and NPPB (*D*) on ATP-induced increment of bumetanide-resistant K^+ influx (*curves 1*) and baseline activity of NKCC (bumetanide-sensitive K^+ influx, *curves 2*) in C7 cells. The cells seeded on regular plastic plates were preincubated for 30 min in 1 ml medium B. Then, the medium was aspirated, and 0.25 ml of medium B with tested compounds was added. After 5 min of incubation at 37°C, 0.25 ml of medium B with 1 μCi/ml ^{86}Rb, and 200 μM ouabain with or without 20 μM bumetanide and 200 μM ATP was added. Isotope uptake was terminated in 10 min. The final concentrations of tested compounds are shown in the X axis. The control values of bumetanide-sensitive and bumetanide-resistant components of K^+ influx were taken as 100%. Means ± SE from 3 experiments performed in quadruplicate are shown.

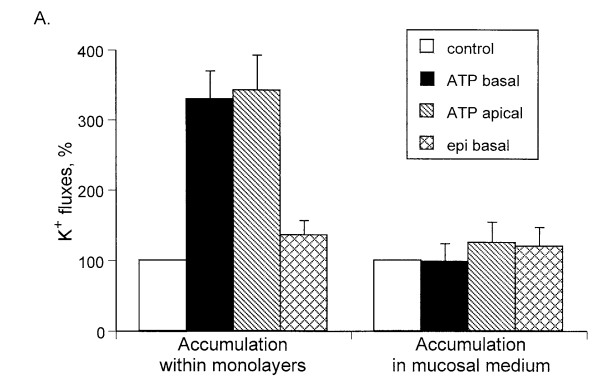
Ca^{2+}-activated Cl^- channels, but does not affect cystic fibrosis transmembrane regulator (CFTR) Cl^- channels and voltage-gated Cl^- channels ClC-2 and ClC-3. NPPB inhibits outwardly rectifying Cl^- channels, CFTR, ClC-2, ClC-5 and ClC-6 but not ClC-3 (Bres et al., 2000; Schultz et al., 2001; Wills & Fong, 2001). Two types of voltage-dependent Cl^- channels of 460 and 46 pS have been identified on the apical and basolateral membranes of MDCK cells (Kolb, Brown & Murer, 1985; Ponce et al., 1991). The effect of NPPB on the activity of these channels has not been studied, whereas it was found that DIDS at a concentration of 1 mM blocked 46 pS Cl^- channels (Ponce et al., 1991). Thus, additional experiments should be carried out to identify basolateral and apical anion channels or other NPBB-sensitive anion transporters involved in P_{2Y}-purinoceptor-triggered Cl^- secretion in C7 cells.

We observed that similarly to a commercially-available high-resistant clone of MDCK cells (Simmons, 1981b; 1990), basolateral NKCC contributes to the sustained phase of ATP-induced I_{SC} in C7 monolayers. This conclusion is supported by partial inhibition of long-lasting charge movement (Q_{SS}) in serosal Na^+-free medium (Fig. 4) and in the presence of bumetanide (Table 2). It is important to underline that treatment of MDCK cells with ATP led to sustained activation of I_{SC} seen after 40 min (Simmons, 1981a,b), whereas in C7-MDCK, I_{SC} was rapidly decreased in 1 min and normalized after 10–15 min of ATP addition (Fig. 2). Our results are in accordance with data obtained with mouse collecting-duct cells (Cuffe et al., 2000). Interestingly, extremely low NKCC activity was observed in C7 monolayers (Table 3) which is in contrast to the 2-fold decrease of ouabain-resistant ^{86}Rb

Table 3. Transport of K^+ (^{86}Rb) from basal-to-apical and from apical-to-basal membranes in C7-MDCK monolayers

Additions	K^+ fluxes from serosal medium, nmol/cm^2/10 min		K^+ fluxes from mucosal medium, nmol/cm^2/10 min	
	Accumulation within monolayer	Accumulation in mucosal medium	Accumulation within monolayer	Accumulation in serosal medium
None (control)	28.2 ± 2.1	3.4 ± 0.6	8.3 ± 0.7	4.4 ± 0.6
Ouabain	12.4 ± 1.3*	3.3 ± 1.0	8.9 ± 0.5	4.8 ± 0.4
Bumetanide	23.8 ± 1.5	3.5 ± 0.3	8.2 ± 0.4	4.3 ± 0.9
Ouabain + bumetanide	12.2 ± 0.9*	3.7 ± 0.8	8.4 ± 0.6	4.8 ± 0.8

To study isotope fluxes from the basal to apical direction, ^{86}Rb was added to serosal medium. To study isotope fluxes from the apical to basal direction, ^{86}Rb was added to mucosal medium. 100 μM ouabain 100 μM bumetanide were added to the same media simultaneously with the isotope. For more details, *see* Methods. Means ± SE for experiments in quadruplicate are given. Asterisk denotes data significantly different from control ($p < 0.001$).



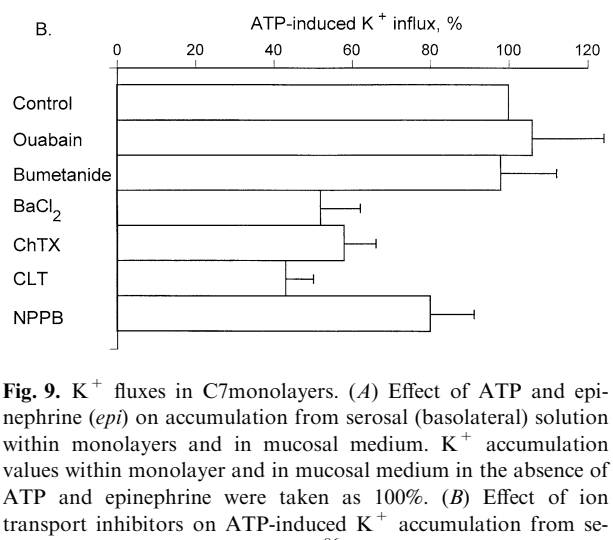


Fig. 9. K^+ fluxes in C7monolayers. (*A*) Effect of ATP and epinephrine (*epi*) on accumulation from serosal (basolateral) solution within monolayers and in mucosal medium. K^+ accumulation values within monolayer and in mucosal medium in the absence of ATP and epinephrine were taken as 100%. (*B*) Effect of ion transport inhibitors on ATP-induced K^+ accumulation from serosal solution within monolayers. ^{86}Rb was added to serosal solution for 10 min. 100 μM ATP, 1 μM epinephrine, 100 μM ouabain, 10 μM bumetanide, 2 μM $BaCl_2$, 0.05 μM ChTX, 2 μM clotrimazole and 100 mM NPPB were added to the basolateral surface of the monolayers simultaneously with isotope. The values of ATP-induced K^+ accumulation within monolayers in the absence of ion transport inhibitors (control) were taken as 100%. Means ± SE from 3 experiments performed in quadruplicate are shown.

influx by furosemide seen in monolayers of MDCK cells (Aiton et al., 1982). The low activity of NKCC in C7 cells is probably caused by relatively high intracellular Cl^- concentration that was only 2-fold less than total content of monovalent cations (Table 4). Apart from elevated NKCC activity, the long-lasting I_{SC} in ATP-treated MDCK cells might be also caused by the presence of intercalated cells. However, ATP-induced I_{SC} in C11 cells was negligible compared to C7 cells, and the presence of up to 50% C11 cells in the monolayer did not affect the kinetics of I_{SC} generated by ATP in C7 cells (Fig. 6).

Activation of Basolateral K^+ Channels Contributes to ATP-Induced Cl^- Secretion

ChTX and CLT are known as potent inhibitors of BK_{Ca} and IK_{Ca} (Rittenhouse et al., 1997; Brugnara, De Franceschi & Alper, 1993). We observed that ATP-induced I_{SC} is partially inhibited by these compounds under their addition to the serosal surface of the monolayers (Table 2). Both BK_{Ca} of 200–300 pS and more frequently IK_{Ca} of 40–60 pS were detected in MDCK cells with the patch-clamp technique (for review, *see* (Lang & Paulmichl, 1995)). Viewed collectively, these data suggest that ATP-induced Cl^- secretion is caused by activation of basolateral BK_{Ca} and(or) IK_{Ca} channels. This hypothesis is in accordance with data obtained in the study of the effect of ATP on unidirectional K^+ fluxes. Indeed, it was shown that ATP augments K^+ efflux across the basolateral membrane of MDCK monolayers (Simmons, 1991b; Tauc, Gastineau & Poujeol, 1993). We observed that ATP sharply elevates K^+ (^{86}Rb) influx through the basolateral surface of C7 monolayers and does not affect transepithelial K^+ fluxes and K^+ uptake from mucosal medium (Fig. 9*A*, Table 5). Moreover, similarly to I_{SC}, ATP-induced K^+ fluxes across the basolateral membrane were partly inhibited by $BaCl_2$, ChTX and CLT (Fig. 9*B*). Elevation of K^+ up to 60 mM in the serosal solution led to inversion of ATP-induced I_{SC} (Figs. 4 and 5), suggesting the involvement of K^+ channels in the induction of Cl^- secretion via the modulation of membrane electrical potential.

Table 4. Effect of ATP and epinephrine on the content of intracellular exchangable K^+, Na^+ and Cl^- monolayers of C7-MDCK cells

Additions	Content of monovalent ions, nmol/cm^2		
	K^+	Na^+	Cl^-
None (control)	174.8 ± 8.1	85.4 ± 11.1	115.3 ± 21.3
ATP, 100 μM	151.0 ± 11.0	92.7 ± 7.9	99.4 ± 6.9
Epinephrine, 1 μM	164.9 ± 10.0	106.3 ± 8.9	114.0 ± 23.6

Cell monolayers were incubated for 3 hr in DMEM containing 2% calf serum, ^{86}Rb, ^{22}Na or ^{36}Cl. ATP and epinephrine were added to the serosal solution for the last 10 min of incubation with isotopes. Means ± SE of experiments performed in triplicate are given.

Table 5. Effect of ATP and epinephrine on K^+ (^{86}Rb) uptake from serosal solutions by monolayers of C7-MDCK cells

Additions	K^+ (^{86}Rb) uptake, nmol/cm^2/10 min
None (control)	26.1 ± 3.4
Ouabain + bumetanide	10.0 ± 0.9
Ouabain + bumetanide + ATP	73.4 ± 6.1
Ouabain + bumetanide + epinephrine	13.1 ± 1.3

100 μM ouabain, 10 μM bumetanide, 100 μM ATP and 1 μM epinephrine were added to the serosal solution simultaneously with ^{86}Rb. Isotope uptake was terminated in 10 min and the content of ^{86}Rb within monolayers was determined as indicated in Methods. Means ± SE of experiments performed in quadruplicate are given.

The data considered above allow us to propose a model of purinergic activation of Cl^- secretion in principal cells (Fig. 10). The activation of P_{2Y}-purinoceptors located on the basolateral or apical surface of the monolayer leads to activation of basolateral ChTX- and CLT-sensitive K^+ channels. These channels differently affect electrical potential of basolateral and apical membranes and generate an electrochemical driving force for transient Cl^- efflux through apical electrogenic anion transporter(s) with low sensitivity to NPPB. In contrast to other types of secretory epithelium, we observed very modest contribution of basolateral NKCC to ATP-induced Cl^- secretion. Because of this, our model suggests that Cl^- influx across the basolateral membrane is mainly mediated by anion channels with high affinity for NPBB.

The proposed model raises several questions. Extensive studies performed with MDCK cells grown on impermeable support demonstrate that P_{2Y} receptors generate diverse signals, including elevation of intracellular Ca^{2+} concentration, activation of MAP kinase and phospholipases A_2, C and D (for reference *see* Gagnon et al., 1999). Do these signaling cascades possess the same efficiency in polarized C7 cells, and which of them play(s) a key role in activation of basolateral K^+ channels? Is the magnitude of modulation of the Cl^- electrochemical driving potential on apical membranes caused by activation of K^+ channels sufficient to generate transcellular Cl^- fluxes, or do P_{2Y}-purinoceptors also modulate the open probability of apical and/or basolateral Cl^- channels by independent mechanism(s)? Which ion transporters mediate ATP-induced K^+ fluxes observed in the presence of ChTX and CLT? We will address these questions in forthcoming studies.

Distinct Ion Transporters Mediate I_{SC} Triggered by Purino- and Adrenoceptors

Apart from purinoceptors, Cl^- secretion in MDCK cells can be induced by activation of adrenoceptors (Brown & Simmons, 1981), muscarinic cholinoceptors (Simmons, 1992), receptors of vasopressin (Lahr et al., 2000), prostaglandin E_1 and E_3 (Simmons, 1991a; Simmons, 1991b). Is the model proposed for agonists of P_{2Y}-purinoceptors (Fig. 10) also applicable to other secretagogues? The involvement of apical anion channels is probably ubiquitous for all of them. Indeed, inhibition of I_{SC} by apical application of 500 μM NPPB was revealed in C7 cells treated with epinephrine (Table 2) and with arginine-vasopressin (Lahr et al., 2000). However, the implication of basolateral K^+ and Cl^- channels seems to be limited to purinoceptor-induced I_{SC}. Indeed, in contrast to ATP, we did not observe any significant effect of basal application of NPBB and inhibitors of K^+ channels on the maximal increment of epinephrine-induced ion current (Table 2). In contrast to ATP, epinephrine only slightly activated K^+ fluxes across the basolateral membrane of C7 cells (Fig. 9*A*, Table 5) and K^+ elevation in serosal solution did not lead to the reverse direction of I_{SC} as noted in ATP-treated cells (Figs. 4 and 5). The molecular mechanisms underlying these features of the regulation of ion fluxes by adreno- and purinoceptors were out of the scope of the present investigation and deserve further analysis.

In conclusion, our results show that transcellular ion current observed in ATP-treated C7 cells resembling principal cells from the collecting duct is only slightly affected by inhibitors of Na^+, K^+, Cl^- cotransport and is mainly caused by Cl^- secretion via the coupled function of apical and basolateral transporters with different sensitivities to the anion channel blocker NPBB. Transcellular Cl^- movement in ATP-treated cells is at least partially caused by activation of basolateral ChTX- and CLT-sensitive K^+ channels.

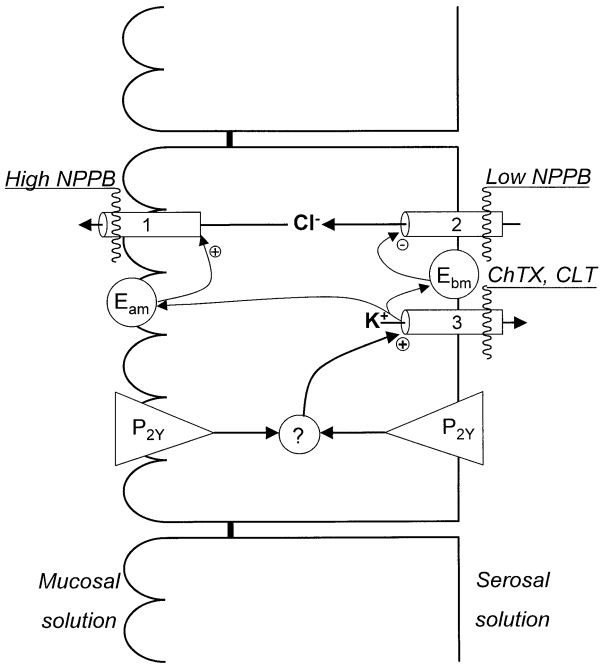


Fig. 10. Possible mechanism of implication of apical and basolateral K^+ and Cl^- channels in transcellular ion current triggered by P_{2Y}-purinoceptors in principal cells. *1* and *2*: Cl^- channels located on apical and basal membranes and inhibited by NPPB at high and low concentrations, respectively; *3*: basolateral K^+ channels inhibited by ChTX and CLT; E_{am} and E_{bm}: electrical potential on apical and basolateral membrane, respectively; + and − signals: leading to activation and inhibition of ion transporters, respectively; ?: + unknown step of signaling cascade. For more details, *see* text.

This study was supported by a grant from The Kidney Foundation of Canada to SNO. The authors acknowledge the editorial assistance of Ovid M. Da Silva, Research Support Office, Research Centre, CHUM.

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

Bailey, M.A., Hillman, K.A., Unwin, R.J. 2000. P_2 receptors in the kidney. *J. Auton. Nerv. Syst.* **81:**264–270

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

Blazer-Yost, B.L., Record, R.D., Oberleithner, H. 1996. Characterization of hormone-stimulated Na^+ transport in a high-resistance clone of the MDCK cell line. *Pfluegers Arch.* **432:**685–691

Bres, V., Hurbin, A., Duvoid, A., Orcel, H., Moos, F.C., Rabie, A., Hussy, N. 2000. Pharmacological characterization of volume-sensitive, taurine permeable anion channels in rat supraoptic glial cells. *Br. J. Pharmacol* **130:**1976–1982

Breyer, M.D., Ando, Y. 1994. Hormonal signaling and regulation of salt and water transport in the collecting duct. *Ann. Rev. Physiol.* **56:**711–739

Brown, C.D.A., Rugg, E.L., Simmons, N.L. 1986. Loop-diuretic inhibition of adrenaline-stimulated Cl^- secretion in a cultured epithelium of renal origin (MDCK). *Quart. J. Exp. Physiol.* **71:** 183–193

Brown, C.D.A., Simmons, N.L. 1981. Catecholamine-stimulation of Cl^- secretion in MDCK cell epithelium. *Biochim. Biophys. Acta* **649:**427–435

Brugnara, C., De Franceschi, L., Alper, S.L. 1993. Inhibition of Ca^{2+}-dependent K^+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidasole derivatives. *J. Clin. Invest.* **92:**520–526

Burnatowska-Hledin, M.A., Spielman, W.S. 1989. Vasopressin V_1 receptors on the principal cells of the rabbit cortical tubule: stimulation of cytosolic free calcium and inositol phosphate production via coupling to a pertussis toxin substrate. *J. Clin. Invest.* **83:**84–89

Chan, C.M., Unwin, R.J., Burnstock, G. 2001. Potential functional roles of extracellular ATP in kidney and urinary tract. *Exp. Nephrol.* **6:**200–207

Cuffe, J.E., Biefeld-Ackermann, A., Thomas, J., Leipziger, J., Korbmacher, C. 2000. ATP stimulates Cl^- secretion and reduces amiloride-sensitive Na^+ absorbtion on M-1 mouse cortical collecting duct. *J. Physiol.* **524:**77–90

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

Giebisch, G. 1998. Renal potassium transport: mechanisms and regulation. *Am. J. Physiol.* **274:**F817–F833

Kirk, K.L. 1988. Binding and internalization of a fluorescent vasopressin analogue by collecting duct cells. *Am. J. Physiol.* **255:**C622–C632

Kolb, H.A., Brown, C.D.A., Murer, H. 1985. Identification of a voltage-dependent anion channel in the apical membrane of a Cl^--secretory epithelium (MDCK). *Pfluegers Arch.* **403:**262–265

Lahr, T.E., Record, R.D., Hoover, D.K., Hughes, C.L., Blazer-Yost, B.L. 2000. Characterization of the ion transport responses to ADH in MDCK-C7 cell line. *Pfluegers Arch.* **439:**610–617

Lang, F., Paulmichl, M. 1995. Properties and regulation of ion channels in MDCK cells. *Kidney Int.* **48:**1200–1205

Mohamed, A., Ferguson, D., Seibert, F.S., Cai, H.-M., Kartner, N., Grinstein, S., Riordan, J.R., Lukacs, G.L. 1997. Functional expression and apical localization of the cystic fibrosis transmembrane conductance regulator in MDCKI cells. *Biochem. J.* **322:**259–265

Moyer, B.D., Loffing, J., Schwiebert, E.M., Loffing-Cueni, D., Halpin, P.A., Karlson, K.H., Ismailov, I.I., Guggino, W.B., Langford, G.M., Stanton, B.A. 1998. Membrane trafficking of the cystic fibrosis gene product, cystic fibrosis transmembrane regulator, tagged with green fluorescent protein in Madin-Darby canine kidney cells. *J. Biol. Chem.* **273:**21759–21768

Orlov, S.N., Dulin, N.O., Gagnon, F., Gekle, M., Douglas, J.G., Schwartz, J.H., Hamet, P. 1999. Purinergic regulation of Na^+, K^+, Cl^- cotransport and MAP kinases is limited to C11-^MDCK cells resembling intercalated cells from collecting ducts. *J. Membrane Biol.* **172:**225–234

Ponce, A., Contreras, R.G., Cereijido, M. 1991. Polarized distribution of chloride channels in epithelial cells. *Cell. Physiol. Biochem.* **1:**160–169

Rittenhouse, A.R., Parker, C., Brugnara, C., Morgan, K.G., Alper, S.L. 1997. Inhibition of maxi-K currents in ferret portal vein smooth muscle cells by the antifungal clotrimazole. *Am. J. Physiol.* 2**73:**C45–C56

Schultz, B.D., Singh, A.K., Devor, D.C., Bridges, R.J. 2001. Pharmacology of CFTR chloride channel activity. *Physiol. Rev.* **79:**S109–S144

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

Simmons, N.L. 1981a. Identification of a purine (P_2) receptor linked to ion transport in a cultured renal (MDCK) epithelium. *Br. J. Pharmacol.* **73:**379–384

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

Simmons, N.L. 1990. Tissue culture of established renal cell lines. *Methods in Enzymology* **191:**426–436

Simmons, N.L. 1991a. Chloride secretion stimulated by prostaglandin E_1 and by forskolin in a canine renal epithelial cell line. *J. Physiol.* **432:**459–472

Simmons, N.L. 1991b. The effect of hypo-osmolarity upon transepithelial ion transport in cultured renal epithelial layers (MDCK). *Pfluegers Arch.* **419:**572–578

Simmons, N.L. 1992. Acetylcholine and kinin augmentation of Cl^- secretion stimulated by prostaglandin in canine renal epithelial cell line. *J. Physiol.* **447:**1–15

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

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

Vander, A.J. 1991. *Renal Physiology*, 5th ed. McGraw-Hill, Inc., New York

Wills, N.K., Fong, P. 2001. ClC chloride channels in epithelia: recent progress and remaining puzzles. *News Physiol. Sci.* **16:**161–166

Woo, J.S., Inoue, C.N., Hanaoka, K., Schwiebert, E.M., Guggino, S.E., Guggino, W.B. 1998. Adenyl cyclase is involved in desensitization and recovery of ATP-stimulated Cl^- secretion in MDCK cells. *Am. J. Physiol.* **274:**C371–C378

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