J. Membrane Biol. 196, 15–31 (2003)
DOI: 10.1007/s00232-003-0621-3

The Journal of
Membrane
Biology

Modulation of Calcium-dependent Chloride Secretion by Basolateral SK4-like Channels in a Human Bronchial Cell Line

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Received: 3 March 2003/Revised: 2 June 2003

Abstract. The human bronchial cell line16HBE14o− was used as a model of airway epithelial cells to study the Ca^{2+}-dependent Cl^- secretion and the identity of K_{Ca} channels involved in the generation of a favorable driving force for Cl^- exit. After ionomycin application, a calcium-activated short-circuit current (I_{sc}) developed, presenting a transient peak followed by a plateau phase. Both phases were inhibited to different degrees by NFA, glybenclamide and NPPB but DIDS was only effective on the peak phase. ^{86}Rb effluxes through both apical and basolateral membranes were stimulated by calcium, blocked by charybdotoxin, clotrimazole and TPA. 1-EBIO, a SK-channel opener, stimulated ^{86}Rb effluxes. Block of basolateral K_{Ca} channels resulted in I_{sc} inhibition but, while reduced, I_{sc} was still observed if mucosal Cl^- was lowered. Among SK family members, only SK4 and SK1 mRNAs were detected by RT-PCR. KCNQ1 mRNAs were also identified, but involvement of K_{cAMP} channels in Cl^- secretion was unlikely, since cAMP application had no effect on ^{86}Rb effluxes. Moreover, chromanol 293B or clofilium, specific inhibitors of KCNQ1 channels, had no effect on cAMP-dependent I_{sc}. In conclusion, two distinct components of Cl^- secretion were identified by a pharmacological approach after a Ca_i^{2+} rise. K_{Ca} channels presenting the pharmacology of SK4 channels are present on both apical and basolateral membranes, but it is the basolateral SK4-like channels that play a major role in calcium-dependent chloride secretion in 16HBE14o− cells.

Key words: CaCC — SK4 — CFTR — KCNQ1 — Bronchial epithelium — 16HBE14o−

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Introduction

A calcium-activated chloride conductance (CaCC) mediating calcium-stimulated chloride secretion has been reported in normal and CF airway epithelia (Knowles, Clarke & Boucher, 1991; Clarke & Boucher, 1992). In the airway of the CFTR (−/−) knockout mouse (Snouwaert et al., 1992), which does not suffer from airway obstruction, the CaCC pathway is preserved and in some regions upregulated (Grubb & Boucher, 1999), suggesting that the CaCC could compensate for the loss of functional CFTR in cystic fibrosis. These data are similar to those previously reported for CF patients (Grubb et al., 1994).

CaCC conductances are functionally expressed in many excitable and non-excitable cells (Arreola et al., 1996; Large & Wang, 1996; Strauss, Wiederholt & Wienrich, 1996; Nilius et al., 1997; Lalevée & Joffre, 1999). Whether the activation of CaCC by a raise in intracellular free calcium (Ca_i^{2+}) involves phosphorylation by the Ca^{2+}/calmodulin-dependent protein kinase II remains controversial (Wagner et al., 1991; Ishikawa, 1996; Arreola et al., 1998). Furthermore, in airway- and colonic-epithelial cells, calcium-dependent chloride secretion requires activation of basolateral K_{Ca} channels and/or cAMP-regulated K^+ channels (K_{cAMP}) (McCann & Welsh, 1990; Devor & Frizzell, 1993; Greger et al., 1997, Mall et al., 2000; Devor, Bridge & Pilenski, 2000, Mall et al., 2003). The efflux of K^+ through K^+ channels hyperpolarizes the apical and basolateral membranes, thereby enhancing the driving force for Cl^- secretion.

The 16HBE14o− cell line has been immortalized from human bronchial epithelium and has been reported to form a differentiated epithelium presenting tight junctions and directional ion transports (Cozens et al., 1994). These cells possess high levels of CFTR mRNA as well as mRNA coding for the α-subunit of

the epithelial sodium channel (ENaC) (Kunzelmann et al., 1996). In addition, the 16HBE14o− cell line presents a Ca^{2+}-dependent activation of chloride and K^+ conductances by several agonists (nucleotides, histamine, bradykinin), resulting in an increase of the transepithelial Cl^- secretion, which was mainly attributed to the activation of the basolateral K^+ channels (Koslowsky et al., 1994). So far, three classes of calcium-activated K_{Ca} channels have been described and classified by their conductances: large (BK), intermediate (IK) and small (SK) conductance calcium-activated K^+ channels (for review, *see* Latorre et al., 1989). These different K_{Ca} channels present distinct pharmacological and electrical characteristics and can therefore be distinguished from each other (Latorre et al., 1989; Garcia et al., 1991; Vergara et al., 1998; Carignani et al., 2002).

The upregulation of CaCC expression and/or activity in secretory epithelia has been proposed as a therapeutic strategy in the treatment of cystic fibrosis (Fuller & Benos, 2000; Pauli et al., 2000). The knowledge of the molecular and cellular mechanisms underlying the regulation of CaCC may suggest potential pharmaceutical targets. In this context, we aimed to 1) investigate the pharmacological characteristics of the calcium-activated chloride conductance and its regulation in the 16HBE14o− cell line and 2) distinguish between a direct regulatory effect of calcium on CaCC and an indirect effect through the opening of basolateral or apical K^+ channels. We also aimed to determine the molecular identity of these K^+ channels potentially involved in the control of Cl^- secretion.

Materials and Methods

Cell Culture

The cell line 16HBE14o− was a generous gift of Dr D.C. Gruenert (Colchester, VT). This cell line derived from bronchial epithelial cells, immortalized by the SV40 T-antigen (Cozens et al., 1994). Cells were cultured on plastic flasks coated with fibronectin (BD Biosciences, MA) collagen (Cohesion, CA) and bovine serum albumin (Sigma, St. Louis, MO). Cells were kept in a humidified atmosphere of 5% CO_2/95% air at 37°C and grown in modified Eagle's medium (MEM, Invitrogen, UK) containing (in g/L): L-glutamine (0.292), D-glucose (1), $NaHCO_3$ (2.2), supplemented with 10% fetal bovine serum (Dominique Dutscher S.A., France). To prevent bacterial contamination a mixture of penicillin and streptomycin (100 U/mL each) was added. For most experiments, 16HBE14o− cells were grown on permeable supports (Transwell-Clear, Corning, NY) coated with fibronectin-collagen, for 8 to 10 days after they reached confluence. Jurkat human leukemia cell line (clone JA3) was a generous gift of Dr. B. Rossi (Nice, France). Jurkat-T cells were cultured in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum (Dominique Dutscher). TE671 human medulloblastoma cell line was a generous gift of Dr. P. Durbec (Marseille, France). TE671 cells were cultured and grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Dominique Dutscher).

Measurements of Short-Circuit Current and Epithelial Resistance

Cell monolayers were mounted in home-made Ussing chambers as described previously (Ehrenfeld, Raschi & Brochiero, 1994). Bath solutions were gassed with 5% CO_2 to adjust the pH to 7.4 and all experiments were performed at 37°C in a humidified atmosphere of 5% CO_2/95% air. The Ussing chamber was connected to an automatic voltage clamp (Physiologic Instruments, VCC-600, TX) and measurements of short-circuit current (I_{sc}) were made in Ringer solution containing (in mM): 120 NaCl, 5 KCl, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ and 11.1 D-glucose. For some experiments, the apical Ringer solution was changed to a Ringer solution with a reduced Cl^- concentration (10 mM) in order to increase the serosal-to-mucosal Cl^- gradient. This solution was obtained by substituting NaCl with equimolar Na-gluconate. In anion-substitution experiments (i.e., Cl^--free solution), equimolar Na-gluconate, K-gluconate and Ca-gluconate replaced NaCl, KCl and $CaCl_2$, respectively. The Ca^{2+} concentration was increased to 5 mM to compensate the Ca^{2+}-buffering capacity of gluconate. In the HCO_3^--free solution, 24.8 mM Na-gluconate replaced $NaHCO_3$ and 10 mM HEPES buffered the solution, the pH being adjusted to 7.4 with 1N NaOH solution. Acetazolamide (1 mM) was added in the HCO_3^--free solution to limit endogenous cell HCO_3^- production. After mounting cell monolayers in the Ussing chamber, an equilibration period of 10 min allowed the stabilization of the resting I_{sc}. The transepithelial resistance (R_t) was measured by applying (1 s) bipolar 1 mV voltage pulses every 60 s and was calculated according to Ohms law ($R_t = \Delta V_t/\Delta I$).

^{86}Rb Effluxes

Cell monolayers grown on permeable supports were loaded with ^{86}Rb (37 kBq/ml) for three hours from the basal side in a humidified atmosphere of 5% CO_2/95% air at 37°C. After three rapid (15 s) washing steps with "cold" Ringer solution, the ^{86}Rb effluxes were followed in open-circuit conditions by sampling the apical and basal bathing solutions at regular time periods. At the end of the experiments, cell monolayers were lysed with a NaOH (1N) solution for four hours in order to measure the remaining radioactivity. Then, the sampled radioactivity was measured after addition of 4 ml liquid scintillation fluid (ACS, Amersham, IL) in a liquid scintillation counter (Packard Instruments). Efflux rates were calculated as the percentage (%) per min of ^{86}Rb loss into the medium relative to total ^{86}Rb contained in the monolayer at the beginning of the time period measured $\{[cpm_x - cpm_{(x+1)}]/cpm_x \times 100$, where x and $x+1$ represent successive time points$\}$ and plotted as a function of time. Due to the large cell K^+ pool, reliable ^{86}Rb efflux measurements could be measured during long time periods; after 10 min of ^{86}Rb efflux, 85% of the initial ^{86}Rb load was still present in non-treated cells, while 29% of the ^{86}Rb load remained in the ionomycin-stimulated cells, with a medium radioactivity at least 20 times larger than the background level.

RNA Isolation and Reverse Transcriptase/Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells grown on permeant supports (Transwell-Clear, Corning, NY) using an RNAeasy Mini Kit (Qiagen, Germany) and was reverse-transcribed using oligo-dT prim-

Table 1. Primers used in RT-PCR to determine expression of SK and KCNQ1 channels in 16HBE14o− cells

Gene	Acc. number		Primer sequence	Position	Tm (°C)	Expected product length (bp)
hSK1*	NM002248	Forward	5′-ACCCCTAAATCTTGGCCATCGT-3′	1962–1983	68.3	281
		Reverse	5′-TAGGCGGGTCCTGCTTTATTCA-3′	2243–2222	68.3	
hSK2*	AF239613	Forward	5′-CGACAAGCACGTCACTTACAA-3′	2098–2118	63.8	213
		Reverse	5′-CTGACATCAGAACCCGGATAA-3′	2311–2291	63.7	
hSK3*	AJ251016	Forward	5′-AATCTCCGATAGCCCCATTG-3′	2472–2491	64.8	310
		Reverse	5′-TCGCTTCCTGTCATCTCCTCTT-3′	2782–2761	66.3	
hSK4	NM002250	Forward	5′-TATGCTGCTATGGACGACCTC-3′	1938–1958	64	277
		Reverse	5′-GATAAGAGCAGAGGCTGGTG-3′	2215–2196	61.5	
hKCNQ1**	AF000571	Forward	5′-CACCATCGAGCAGTATGCCGC-3′	539–559	71.9	436
		Reverse	5′-CATCGCGTCCTTCTCAGCCA-3′	974–955	71	
hb-actin	XOO351	Forward	5′-CTGTGCTATCCCTGTACGCCTC-3′	413–434	66.8	436
		Reverse	5′-CATGATGGAGTTGAAGGTAGTTTCG-3′	849–825	66.4	

hSK1-hSK4, human small-conductance K^+ channels; hKCNQ1, human voltage-gated K^+ channel.
*Carignani et al., 2002; **Bertaso et al., 2002.

ers during 1 h at 37°C (Superscript First-Strand cDNA Synthesis System for RT-PCR, Invitrogen). The primer sequences and expected product length for hSK1, hSK2, hSK3, hSK4, hKCNQ1 and hβ-actin are given in Table 1. For all PCR reactions: denaturation 94°C/30 s; annealing 60°C/30 s; extension 72°C/30 s; 30 and 40 cycles; recombinant Taq DNA polymerase was from Invitrogen. PCR products were analyzed on a 1.7% agarose gel using double-stranded DNA fragments from 100 bp to 12 Kb as standard (1 Kb Plus DNA Ladder, Invitrogen). After purification (QIAquick gel extraction kit, Qiagen), the sequences of the PCR products were confirmed by sequencing (Genome express, Meylan, France).

Chemicals

Chloride-channel blockers: DTT (Cleland reagent racemic; (±) threo-1,4-Dimercapto-2,3-butanediol), DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid, disodium salt), glybenclamide (N-p-{2-(5-chloro-2-methoxybenzamido)ethyl}benzenesulfonyl-N′-cyclohexylurea), NFA (2-(3-[trifluoromethyl]anilino)nicotinic acid) and NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid) were purchased from Sigma. Potassium-channel blockers: TPA (tetrapentylammonium bromide) was purchased from Fluka (Fluka Chemie, Switzerland), chromanol 293B was a generous gift from Dr. J. Pünter (Aventis Pharma Deutschland GmbH, Germany) and clotrimazole (1-(*o*chloroα,αdiphenylbenzyl)imidazole), charybdotoxin, apamin and iberiotoxin were purchased from Sigma). Others: ionomycin, pCPT-cAMP (8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate), IBMX (3-isobutyl-1-methylxanthine), H-89, chelerythrine chloride, 1,9-dideoxyforskolin, acetazolamide, clofilium tosylate and ATP (adenosine 5′-triphosphate) were purchased from Sigma. 1-EBIO was purchased from Tocris (Ellisville, MO). All reagents were prepared as >1000-fold stock solutions. TPA, charybdotoxin, iberiotoxin and ATP were dissolved in water. Apamin was dissolved in a 0.05 M acetic acid solution and all other chemicals in DMSO.

Data Analysis

All data are presented as mean ± SEM, where n indicates the number of experiments. Paired or unpaired Student's t-test was used and a P value <0.05 was chosen to indicate statistical significance. Statistical analysis was done by the GraphPAD software version 1.13 (Christiane Mo, University of Montreal, Canada).

Results

Characterization of the Calcium-activated Chloride Conductance

16HBE14o− cells grown on permeant supports form a polarized and tight monolayer. In resting conditions, cell monolayers displayed a transepithelial resistance (R_t) of 1332 ± 156 Ωcm^{-2} (n = 42) and a short-circuit current (I_{sc}) of 0.9 ± 0.1 μA cm^{-2} (n = 42); I_{sc} (and R_t) were not affected by apical application of 100 μM amiloride (*data not shown*), consistent with the report of Koslowsky et al. (1994) where the presence of an apical sodium conductance was found to be dependent on culture conditions. Increasing Ca_i^{2+} by addition of 0.2 μM ionomycin to the apical solution enhanced I_{sc} and simultaneously reduced R_t (Fig. 1*A*). A transient peak (I_{peak}, 43.2 ± 1.6 μA cm^{-2}, n = 42) occurring in less than one minute was followed by a plateau phase ($I_{plateau}$, 5.2 ± 0.2 μA cm^{-2}, n = 42) that stabilized after 6–8 minutes of drug application. The value found for the peak and the plateau phase were significantly different from the resting I_{sc} value (P < 0.001). Since no sodium transport could be detected, it was likely that the observed conductance was due to anion secretion.

Chloride or bicarbonate was substituted by gluconate or chloride, respectively, in the experimental medium to distinguish between these anions (Table 2). When using Cl^--free solutions, I_{peak} was reduced by 79% and the peak phase was not followed by a plateau phase. HCO_3^- substitution, in the presence of 1 mM acetazolamide, slightly affected the ionomycin-induced currents. I_{peak} was reduced by 7% and $I_{plateau}$ was reduced by 14% (Table 2). This indicates that the Ca^{2+}-activated I_{sc} mainly corresponds to Cl^- secretion.

The peak and plateau phases observed in the calcium-induced I_{sc} response may correspond to two

Table 2. Effect of anion substitution on the ionomycin-stimulated I_{sc}

	Peak phase ΔI_{sc} ($\mu A\ cm^{-2}$)	Plateau phase ΔI_{sc} ($\mu A\ cm^{-2}$)	n
(Cl^-/HCO_3^-)-containing medium	30.0 ± 3.9	4.5 ± 0.9	7
Cl^-- free medium	6.4 ± 0.7	0.2 ± 0.1	7
Comparison of means	$P < 0.001$	$P < 0.001$	
(Cl^-/HCO_3^-)-containing medium	33.6 ± 2.1	4.9 ± 0.3	13
(HCO_3^-/CO_2)-free medium + acetazolamide	31.1 ± 2.7	4.2 ± 0.2	13
Comparison of means	$P < 0.05$	$P < 0.001$	

Cell monolayers were incubated 30 min with defined media on both sides before 0.2 μM ionomycin application. The (Cl^-/HCO_3^-)-containing medium and the Cl^--free medium were gassed with 5% CO_2/95% air while the (HCO_3^-/CO_2^-)- free medium contained 1 mM acetazolamide and was gassed with air.

different conductances leading to Cl^- secretion or to only one conductance, which partly inactivates. A pharmacological approach was therefore designed to characterize the ionomycin-induced I_{sc} response and to distinguish between these two possibilities. DTT (2 mM), DIDS (500 μM), glybenclamide (100 μM), NFA (100 μM) or NPPB (100 μM) were added to the apical bathing solution either before or after ionomycin application. As shown in Fig. 1*B*, I_{peak} was blocked by DIDS (29.6 ± 2.9%, $n = 7$, $P < 0.001$), glybenclamide (51.8 ± 2.0%, $n = 4$, $P < 0.001$), NFA (86.5 ± 8.2%, $n = 3$, $P < 0.005$) and NPPB (81.9 ± 3.5%, $n = 3$, $P < 0.005$), but was not affected by DTT (−19.3 ± 10% $n = 3$, n.s.). $I_{plateau}$ (Fig. 1*C*) was inhibited by glybenclamide (83.7 ± 4.6%, $n = 13$, $P < 0.001$), NFA (54.9 ± 8.0%, $n = 8$, $P < 0.001$) and NPPB (107.0 ± 6.8%, $n = 4$, $P < 0.001$) but not by DIDS (8.3 ± 4.0%, $n = 6$, n.s.) and DTT (3.0 ± 22.0%, $n = 3$, n.s.). $I_{plateau}$ inhibition by NPPB or glybenclamide but not by NFA, was mostly reversible. $I_{plateau}$ recovered by 77.4 ± 5.1%, $n = 3$, 63.0 ± 6.2%, $n = 7$, and 17.6 ± 7.6%, $n = 3$ after washout of NPPB, glybenclamide, and NFA, respectively. NFA may enter the cells and act from the internal side. Therefore, I_{peak} and $I_{plateau}$ present different percentages of drug inhibition (the statistical evaluation of the difference in drug sensitivity of ΔI_{peak} and $\Delta I_{plateau}$ gave $P < 0.005$ for glybenclamide or DIDS, $P < 0.05$ for NPPB or NFA and n.s. for DTT) suggesting that two different Cl^- conductances may be activated upon ionomycin application.

The activation of CFTR by PKC has been reported in CHO and C127 cells stably expressing the cystic fibrosis gene (Tabcharani et al., 1991; Dechecchi et al., 1993) and in human airway (Paradiso, Ribeiro & Boucher, 2001). Considering the sensitivity of the plateau phase to glybenclamide and its insensitivity to DIDS, pointing to a possible involvement of CFTR in the calcium-stimulated current, we investigated the effect of chelerythrine, a specific PKC- but broad-spectrum-inhibitor of PKC isozymes (Herbert et al., 1990). Pretreatment of the 16HBE14o− cells with 4 μM chelerythrine (basal side, one hour application) slightly inhibited the ionomycin-stimulated current. The ΔI_{peak} were of 27.5 ± 0.8 $\mu A\ cm^{-2}$ and 33.8 ± 1.0 $\mu A\ cm^{-2}$ with or without chelerythrine, respectively ($n = 8$), $P < 0.001$. $\Delta I_{plateau}$ were of 3.5 ± 0.4 $\mu A\ cm^{-2}$ and 4.2 ± 0.4 $\mu A\ cm^{-2}$ ($n = 8$; means not significantly different) in presence or in absence of the PKC inhibitor.

Presence of K_{Ca} Channels in 16HBE14o− Cells

The rise in Ca_i^{2+} may modulate the apical anion channels, either directly or through a calcium-sensitive signaling pathway, and/or affect calcium-activated K^+ channels (K_{Ca}) that generate the electrochemical driving force for Cl^- exit at the apical membranes. Basolateral K_{Ca} channels have been reported to play a role in the control of the apical membrane potential in several transporting epithelia such as intestine (for recent review, *see* Barrett & Keely, 2000), renal tubule (Paulmichl et al., 1991) and airways (Smith & Frizzell, 1984; McCann & Welsh, 1990; Devor et al., 1996). Therefore, we investigated the presence of K_{Ca} channels in 16HBE14o− cells.

We assessed the K^+ permeability after increasing Ca_i by measuring ^{86}Rb effluxes through apical and basolateral membranes of filter-grown 16HBE14o− cells. Intracellular calcium was increased either by apical application of ionomycin or ATP. This nucleotide has been reported to increase Ca_i through activation of purinergic receptors (Sienaert et al., 1998; Walsh, Harrey & Urwald, 2000) that subsequently stimulate a Cl^- conductance (Mason, Paradiso & Boucher, 1991; Koslowsky et al., 1994) in these cells.

As illustrated in Fig. 2*A*, ionomycin application increased the ^{86}Rb effluxes through basolateral membranes. This stimulation was observed 30 s after drug addition, reached a maximum effect after 90 s (12-fold increase) and decreased progressively to a level 6 times higher than the control level 5 min after ionomycin application. Apical ATP application (Fig. 2*B*) also stimulated ^{86}Rb effluxes through basolateral

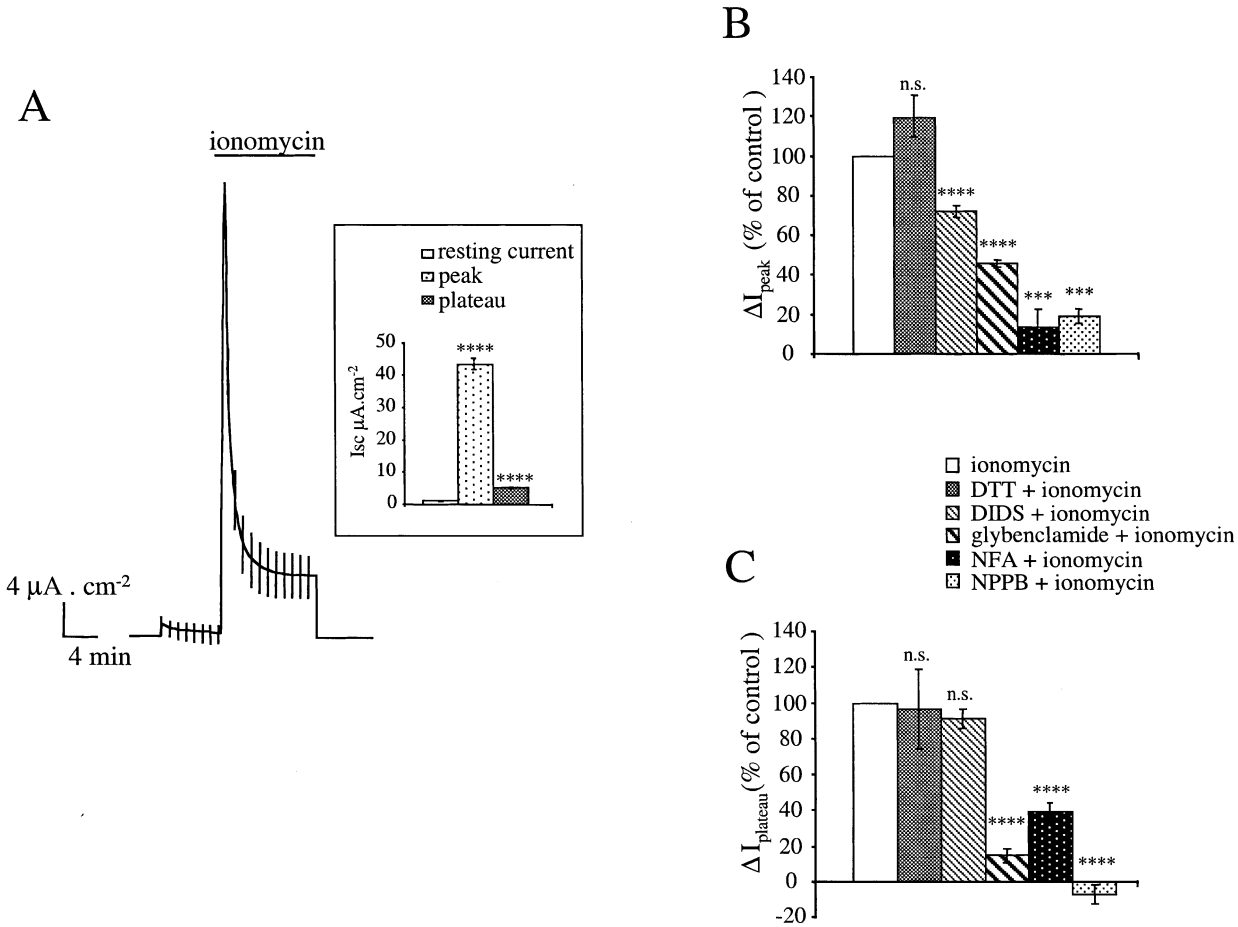

Fig. 1. Ionomycin induces a biphasic stimulation of I_{sc} in 16HBE14o- cells. (*A*) Ionomycin (0.2 µM) application (apical side) induces a fast I_{sc} increase (I_{peak}) followed by a plateau phase ($I_{plateau}$). Mean ± SE of the resting current; the peak and plateau phases are given in the inset. ****$P < 0.001$ compared with resting currents, Student's *t*-test; $n = 42$. (*B*) and (*C*) Pharmacology of I_{peak}, and $I_{plateau}$, respectively. Effect of 2 mM DTT ($n = 3$), 500 µM DIDS ($n = 7$), 100 µM glybenclamide ($n = 4$), 100 µM NFA ($n = 3$) and 100 µM NPPB ($n = 3$) on I_{peak} (*B*). Effect of 2 mM DTT ($n = 3$), 500 µM DIDS ($n = 6$), 100 µM glybenclamide ($n = 13$), 100 µM NFA ($n = 8$) and 100 µM NPPB ($n = 4$) on $I_{plateau}$ (*C*). Ordinates correspond to the ionomycin-stimulated current (ΔI_{peak} or $\Delta I_{plateau}$) in control or after 15 min drug application. Mean ± SE are given in %. Significance by Student's *t*-test: **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$ in comparison with control, n.s. indicates not significant. Significance of the difference in drug sensitivity of ΔI_{peak} and $\Delta I_{plateau}$ was $P < 0.005$ for glybenclamide, $P < 0.005$ for DIDS, $P < 0.05$ for NPPB, $P < 0.05$ for NFA and n.s. for DTT.

membranes, but the effect was smaller (a maximum 5.5-fold increase was obtained at 60 s) and less sustained (^{86}Rb efflux rates returned to control levels after 5 min of stimulation). The ^{86}Rb effluxes remained constant in control experiments (lower traces of Fig. 2*A* and *B*).

As reported in Fig. 2*C* and *D*, a moderate but significant K^+ permeability also exists at the apical membranes of the 16HBE14o− cells. Comparing apical and basolateral ^{86}Rb efflux rates on same monolayers and with same time periods (*see* Fig. 4 for illustration) gave a 3.5- to 5-fold larger basolateral efflux rate relative to the apical one. Ionomycin and ATP both increased the ^{86}Rb effluxes through the apical membranes to the same degree (twofold increase). As observed with basolateral ^{86}Rb effluxes (*see* above), the stimulation was transient following ATP application but sustained following ionomycin application. These different time courses correlate with the Ca_i^{2+} changes occurring after ionomycin or ATP application (*data not shown*). Thus, K_{Ca} channels are present on both membranes of polarized 16HBE14o− cells and are stimulated by elevation of Ca_i^{2+} induced by ATP or ionomycin application.

Pharmacological Characterization of Apical and Basolateral K_{Ca} Permeabilities

We further determined the pharmacological profiles of the K_{Ca} channels present on the apical and basolateral membranes.

Apical application of 50 nM charybdotoxin, 10 µM clotrimazole or 1 mM TPA blocked the ionomycin-stimulated ^{86}Rb effluxes through apical mem-

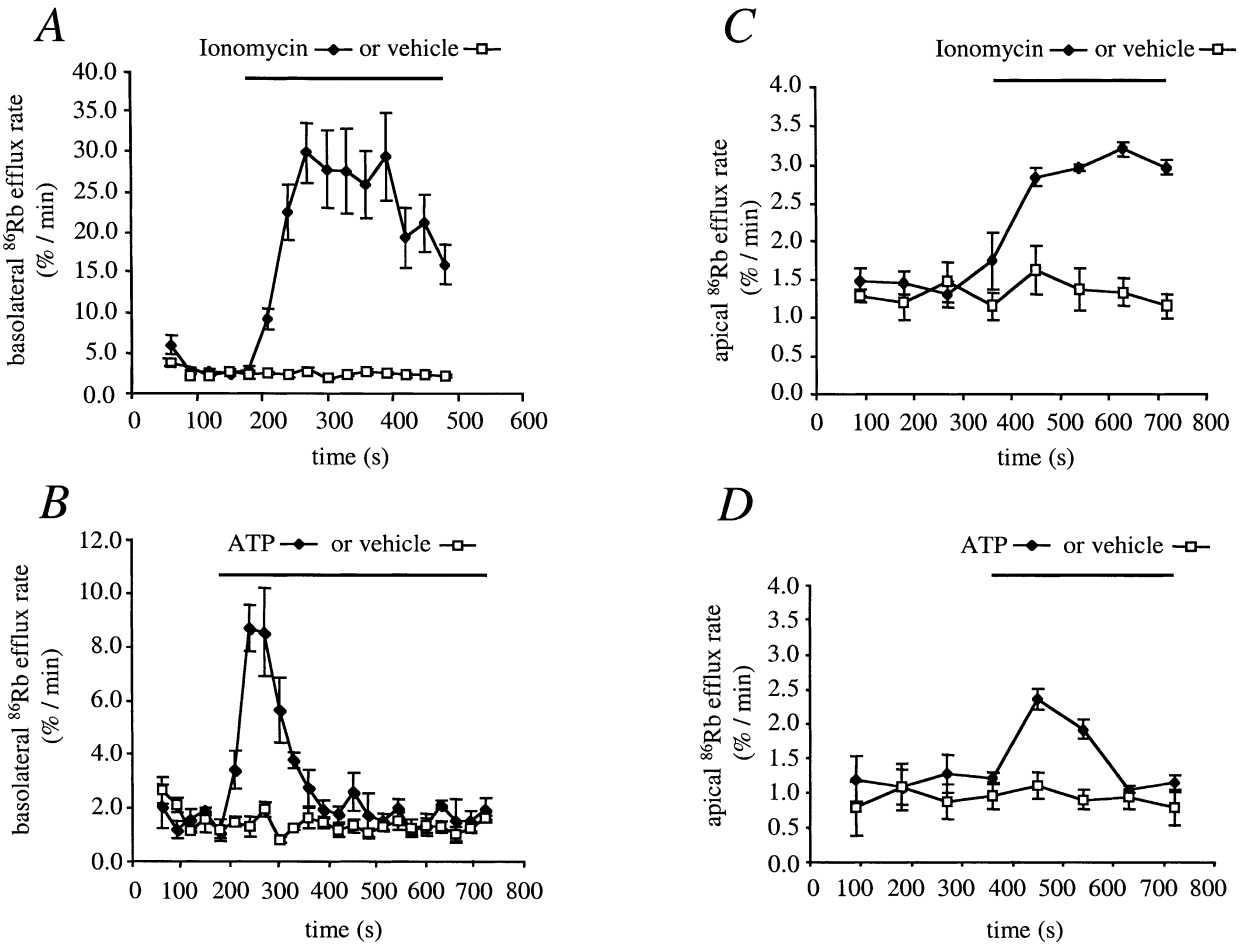

Fig. 2. Effect of ionomycin or ATP on ^{86}Rb efflux rates through apical and basolateral membranes in 16HBE14o− cells. Ordinates report ^{86}Rb efflux rates measured through basolateral (*A*, *B*) or apical membranes (*C*, *D*). Efflux was calculated as the percentage (%) per min of ^{86}Rb loss into the medium relative to total ^{86}Rb contained in the monolayer at the beginning of the measured time period. Ionomycin (0.2 μM) or ATP (100 μM) was added after ^{86}Rb efflux stabilization. In Fig. 2*A*, $n = 10$ and $n = 3$ for ionomycin and vehicle experiments, respectively; in Fig. 2*C*, $n = 6$ and $n = 4$ for ionomycin and vehicle experiments, respectively; in Fig. 2*B*, $n = 3$ for both, ATP and vehicle experiments; in Fig. 2*D*, $n = 4$ and $n = 3$ for ATP and vehicle experiments, respectively.

branes by 52.9% ($P < 0.01$, $n = 4$), 92.8% ($P < 0.001$, $n = 4$) and 43% ($P < 0.01$, $n = 6$), respectively. Apamin (100 nM) produced a moderate inhibition ($P < 0.005$, $n = 3$) and 100 nM iberiotoxin had no effect (Fig. 3*A*). Basal application of charybdotoxin, clotrimazole or TPA blocked the ionomycin-stimulated ^{86}Rb effluxes through basolateral membranes by 63.3% ($P < 0.001$, $n = 4$), 97.3% ($P < 0.001$, $n = 4$) and 87% ($P < 0.001$, $n = 4$), respectively, but iberiotoxin or apamin had no effect (Fig. 3*B*). Since clotrimazole was found to block SK4 channels with an $IC_{50} < 100$ nM (Ishii et al., 1997; Warth et al., 1999) and SK2 and SK3 channels with an $IC_{50} > 20$ μM (Wulff et al., 2000), we tested the effect of a 200 nM concentration of clotrimazole on the ionomycin-stimulated ^{86}Rb effluxes. The inhibitory effect of 200 nM clotrimazole was 65.1 ± 2.6%, $n = 4$, $P < 0.001$ for ionomycin-stimulated ^{86}Rb effluxes through apical membranes and 74.6 ± 2.8%, $n = 4$, $P < 0.001$ for ionomycin-stimulated ^{86}Rb effluxes through basolateral membranes, indicating an IC_{50} lower than 200 nM.

The ATP-stimulated ^{86}Rb effluxes through apical and basolateral membranes presented a pharmacological profile similar to that found with ionomycin application. Clotrimazole application induced a 77.5% inhibition ($P < 0.005$, $n = 4$) and an 84.3% inhibition ($P < 0.005$, $n = 4$) of ATP-stimulated ^{86}Rb effluxes through apical and basolateral membranes respectively (Fig. 4*A* and *B*). Charybdotoxin also inhibited the ATP-stimulated ^{86}Rb effluxes through apical and basolateral membranes by 48.4% ($P < 0.05$, $n = 4$) and 40.6% ($P < 0.01$, $n = 4$), respectively. Apamin and iberiotoxin had no effect on the ATP-stimulated ^{86}Rb effluxes through either cell membrane (*data not shown*).

The pharmacological profile of the calcium-stimulated K^+ permeability in 16HBE14o− cells strongly

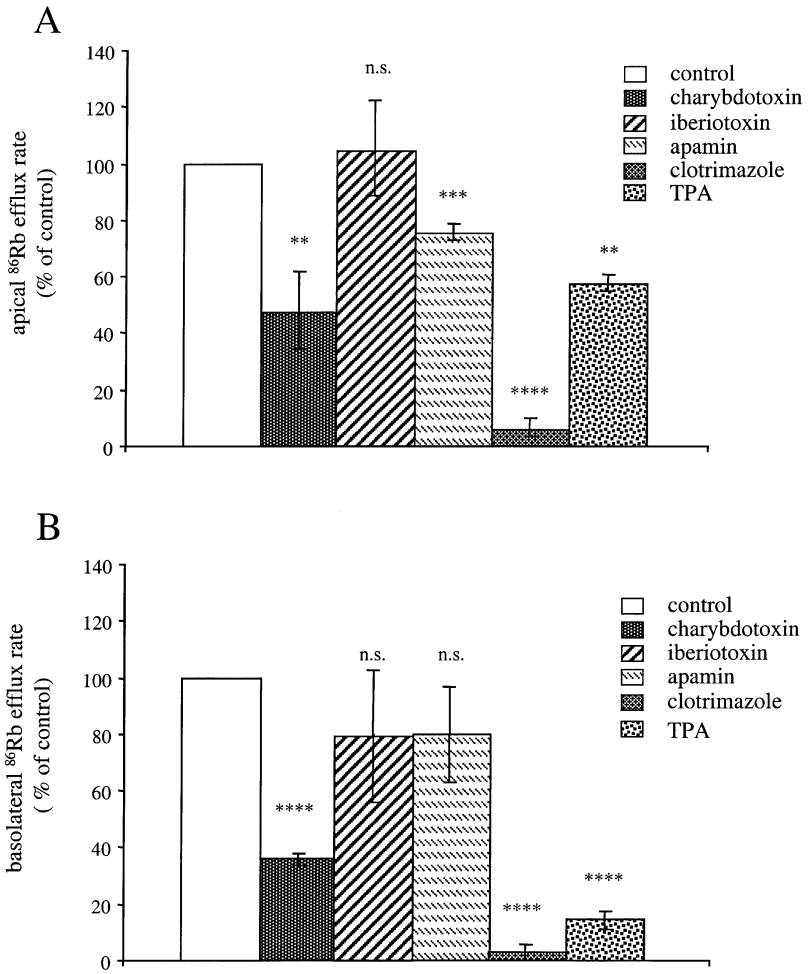

Fig. 3. Pharmacology of the ionomycin-stimulated ^{86}Rb efflux rates in 16HBE14o− cells. ^{86}Rb efflux rates through apical (*A*) or basolateral membranes (*B*). Ordinates correspond to the % of ionomycin-stimulated ^{86}Rb efflux rates, in the presence of a K_{Ca} blocker (maximal effect in absence of K_{Ca} blocker taken as reference). Ionomycin (0.2 μM) was added to the apical bathing solution. Charybdotoxin (50 nM; $n = 4$), iberiotoxin (100 nM; $n = 3$), apamin (100 nM; $n = 3$), clotrimazole (10 μM; $n = 4$) and TPA (1 mM; $n = 6$ and $n = 4$ for apical and basolateral membranes, respectively) were added on both sides. Significance by Student's *t*-test: $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$ compared with control.

resembles that described for the SK family (*see* Discussion). To further explore the possible involvement of these channels, we used 1-ethyl-2-benzimidazolinone (1-EBIO), which is known to activate the Ca^{2+}-activated SK4 K^+ channel in intestinal epithelial cells (Hamilton, Meads & Butt, 1999; Warth et al., 1999), in parotid acinar cells (Takahata, Hayashi & Ishikawa, 2003) and the heterologously expressed SK4/IK1 channel (Pedersen et al., 1999; Syme et al., 2000; Singh et al., 2001; von Hahn et al., 2001). 1-EBIO has also been reported to open SK1 and SK2 channels (Pedarzani et al., 2001). In 16HBE14o− cells, 1 mM 1-EBIO (added on both sides) induced a 3.5-fold increase of ^{86}Rb effluxes through both apical and basolateral membranes (Fig. 4*C* and *D*) after 2 or 4 minutes of 1-EBIO application, respectively. Clotrimazole (10 μM) application (both sides) totally blocked the 1-EBIO-stimulated ^{86}Rb effluxes.

Taken together our present results show that the pharmacological profile of K_{Ca} channels in 16HBE14o− cells correspond to that of SK channels and in particular to that described for the calcium-dependent K^+ channel SK4 (Joiner et al., 1997; Warth et al., 1999).

Key Role of K_{Ca} Channels in Calcium-activated Chloride Secretion

We attempted to assess the contribution of basolateral K_{Ca} channels to the calcium-stimulated Cl^- secretion by testing TPA and clotrimazole, two K_{Ca} blockers that strongly inhibited the ^{86}Rb permeability in 16HBE14o− cells. The ionomycin-induced increase in Cl^- conductance was largely blocked by 10 μM clotrimazole or by 1 mM TPA application added to the basolateral medium (Fig. 5*A*). I_{peak} inhibition was of 80.2 ± 2.7% ($n = 4$, $P < 0.001$) and of 90.4 ± 4.0% ($n = 4$, $P < 0.001$), while that of $I_{plateau}$ was of 103.1 ± 7.2% ($n = 4$, $P < 0.001$) and of 115.0 ± 5.6% ($n = 4$, $P < 0.001$) for clotrimazole and TPA, respectively. These data indicate a major contribution of K_{Ca} channels to the calcium-induced chloride secretion, probably through the generation of a favorable driving force for apical Cl^- exit.

We next determined whether Ca_i^{2+} has also an effect on Cl^- channels. In this aim, we generated a favorable gradient for Cl^- exit by lowering the apical Cl^- concentration. This procedure should reduce the contribution of K_{Ca} channels on the establishment of a

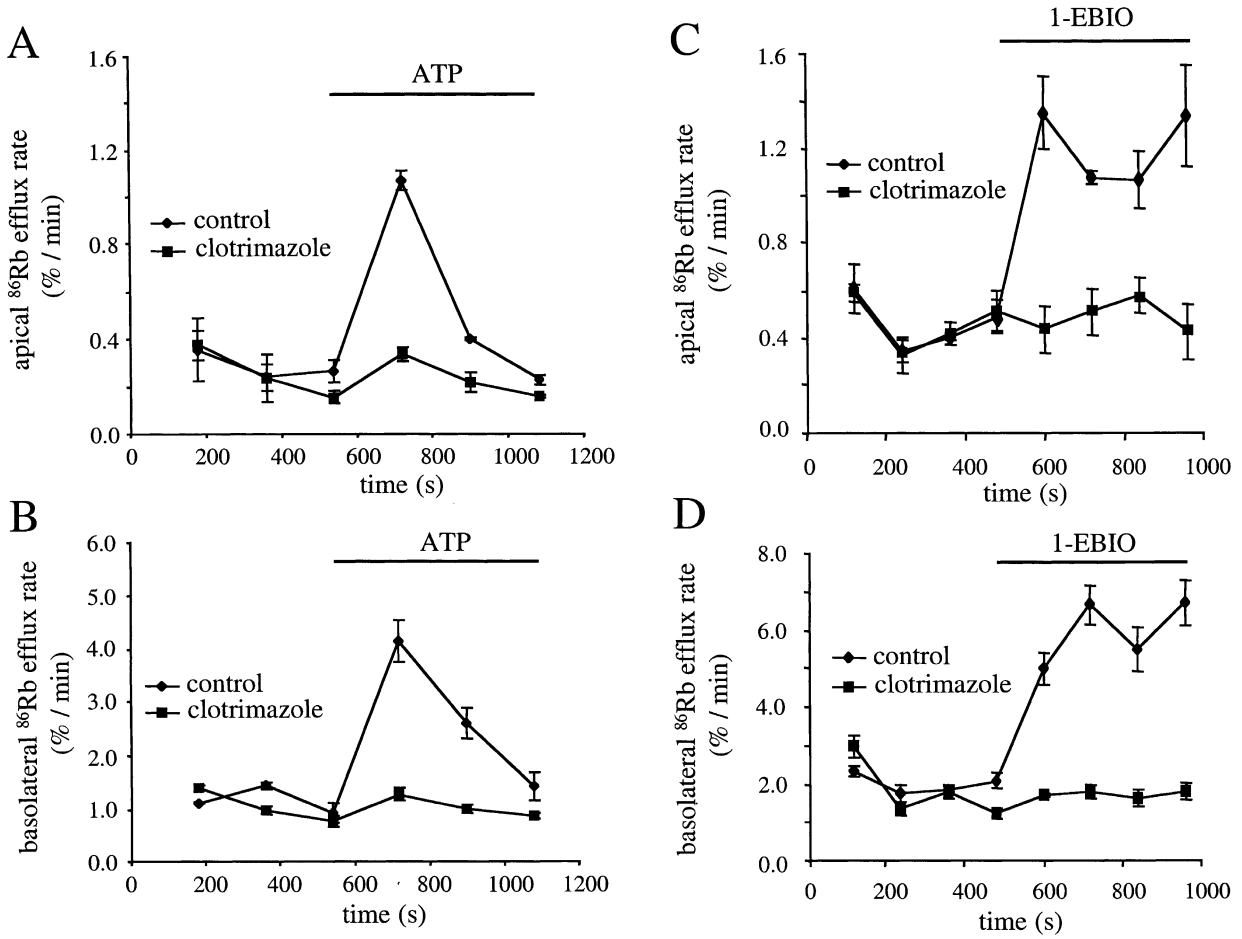

Fig. 4. Inhibitory effect of clotrimazole on apical and basolateral ^{86}Rb efflux rates stimulated by ATP or by 1-EBIO. ^{86}Rb efflux rates through apical (*A*, *C*) and basolateral (*B*, *D*) membranes. ATP (100 μM) was applied to the apical side of the monolayer, while 1-EBIO (1 mM) and clotrimazole (10 μM) were applied on both sides. For all experiments, $n = 4$.

favorable driving force for apical Cl^- exit and therefore we expected K_{Ca} channel blockers to be less effective (Fig. 5*B*). In presence of a Cl^- gradient, I_{peak} inhibition was 57.6 ± 2.5% ($n = 4$, $P < 0.001$) and 71.5 ± 1.9% ($n = 4$, $P < 0.001$), while that of $I_{plateau}$ was 67.5 ± 4.8% ($n = 4$, $P < 0.005$) and 26.6 ± 9.8% ($n = 4$, $P < 0.05$) for clotrimazole and TPA, respectively. Statistical evaluation of the difference of I_{peak} inhibition, in the presence or absence of a Cl^- gradient, gave a P value < 0.005 for clotrimazole and a P value < 0.05 for TPA. For $I_{plateau}$ the statistical evaluation gave P values < 0.05 and < 0.005 for clotrimazole and TPA, respectively. The blocking effects of these inhibitors on I_{peak} and $I_{plateau}$ were therefore reduced when apical Cl^- concentration was lowered. These data confirm that K_{Ca} channels function in Cl^- secretion through the generation of the driving force and suggest that calcium may have an effect on the apical Cl^- conductance distinct from that on K_{Ca} conductance.

We next investigated the effect of blocking apical K_{Ca} channels on the ionomycin-induced I_{sc} ($I_{plateau}$) by applying apamin (100 nM) or charybdotoxin (100 nM) on the apical membrane. Apical apamin or charybdotoxin application induced an instantaneous (maximum in 15–20 s) but transient increase in $I_{plateau}$ (maximum increase of $I_{plateau}$: 1.3 ± 0.2 μA cm^{-2}, $n = 5$, $P < 0.001$ and 1.4 ± 0.4 μA cm^{-2}, $n = 4$, $P < 0.05$ for apamin and charybdotoxin, respectively). This transient stimulation of $I_{plateau}$ may reflect a block of apical K_{Ca} channels, unmasking the dominant Cl^- current. However, $I_{plateau}$ further stabilized (in 4 min) to values not different from those obtained in non-treated monolayers (difference in $I_{plateau}$ between toxin-treated and non-treated monolayers: 0.01 ± 0.15 μA cm^{-2}, $n = 5$, n.s. and 0.33 ± 0.16 μA cm^{-2}, $n = 4$, n.s. for apamin and charybdotoxin, respectively). Therefore, the contribution of apical K_{Ca} channels to the ionomycin-induced I_{sc} ($I_{plateau}$) is rather minor, suggesting that we are mainly dealing with currents related to Cl^- secretion.

CPT-cAMP Does not Activate K^+ Channels in 16HBE14o− Cells

Recent reports have provided evidence of the contribution of the KCNQ1 channel located on baso-

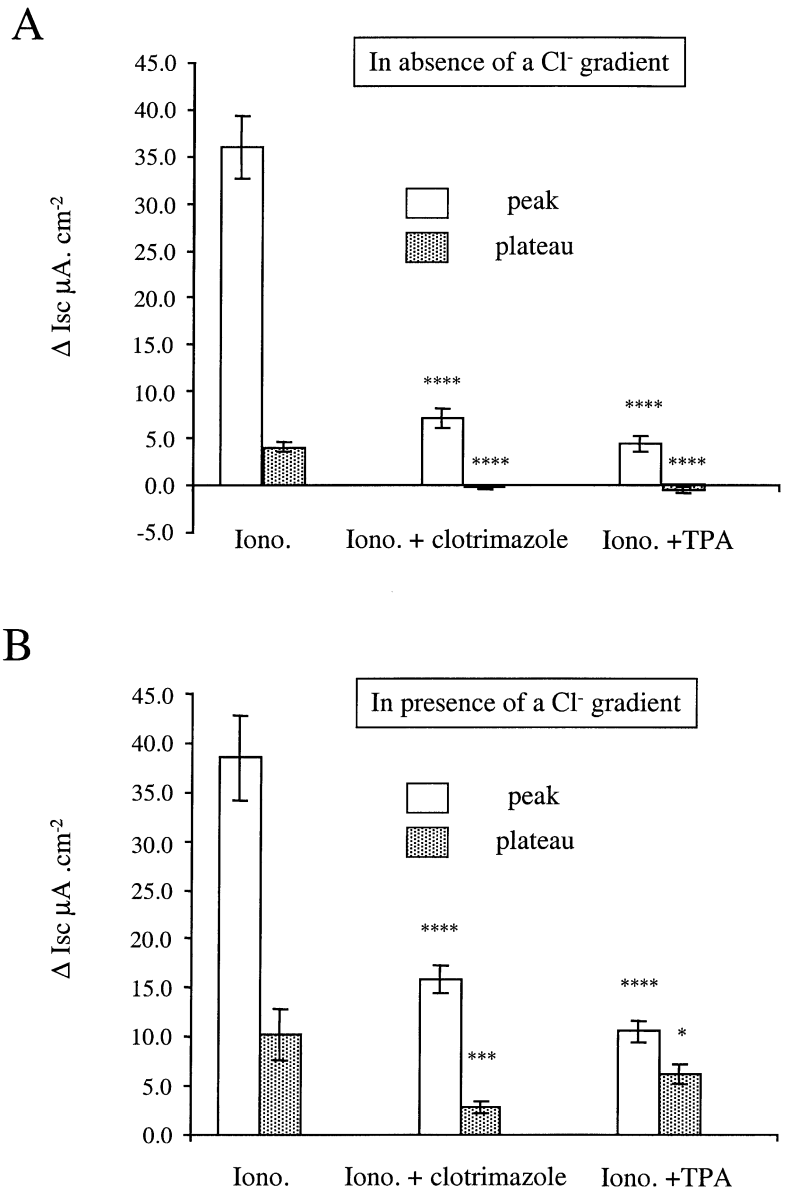

Fig. 5. Effects of clotrimazole and TPA on ionomycin-stimulated currents (*A*) No transepithelial Cl^- gradient. Clotrimazole (10 µM) or TPA (1 mM) were added to the basolateral bathing solution 10 min before ionomycin (0.2 µM) application. (*B*) Application of a transepithelial basolateral-to-apical Cl^- gradient (130 mM/10 mM). Clotrimazole (10 µM) or TPA (1 mM) were added to the basolateral side 10 min before ionomycin (0.2 µM) application. Significance by Student's *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.001$ compared with control; $n = 4$ for all experiments.

lateral membranes of airway epithelia to the driving force for the cAMP-dependent anion secretion (Bleich et al., 1997; Mall et al., 2000, 2003; Grahammer et al., 2001). We first investigated in 16HBE14o− cells, the possible involvement of K_{cAMP} channels in the cAMP-dependent Cl^- secretion using chromanol 293B or clofilium, reported to be blockers of KCNQ1 channels (Bleich et al., 1997; Honoré et al., 1991; Warth & Bleich, 2000; Cowley & Linsdell, 2002). For this purpose we lowered the apical Cl^- concentration to generate a Cl^- gradient and we applied CPT-cAMP (serosal side). The resting I_{sc} in absence of a Cl^- gradient was 1.2 ± 0.7 µA cm^{-2}, $n = 7$. In presence of a Cl^- gradient, and as expected from opening CFTR channels, CPT-cAMP stimulated I_{sc} (Fig. 6*B*) from 16.5 ± 0.6 µA cm^{-2} to 29.0 ± 1.1 µA cm^{-2} ($n = 4$) after 7 min of CPT-cAMP application. Furthermore, the CPT-cAMP stimulated I_{sc} was totally blocked (103.1 ± 11.3% of inhibition, $n = 3$, $P < 0.05$) by a one-hour-on-both-sides pretreatment of the cells with 10 µM H-89, a PKA inhibitor (Chijiwa et al., 1990). The CPT-cAMP stimulation was not observed in the absence of a Cl^- gradient (Fig. 6*A*), confirming previous observations interpreted by assuming Cl^- at eguilibrium at the apical membrane of 16HBE14o− cells (Cozens et al., 1994). We can also point out that the increase in I_{sc} associated with the establishment of a Cl^- gradient reflects mainly constitutively active cell Cl^- conductances since the current was blocked (74.3 ± 7.1% inhibition, $n = 5$) by subsequent application of 100 µM NPPB, 500 µM DIDS, 100 µM glybenclamide or 100 µM NFA. As illustrated in Fig. 6*B*, the CPT-cAMP-dependent I_{sc} was not affected by 100 µM chromanol 293B applied

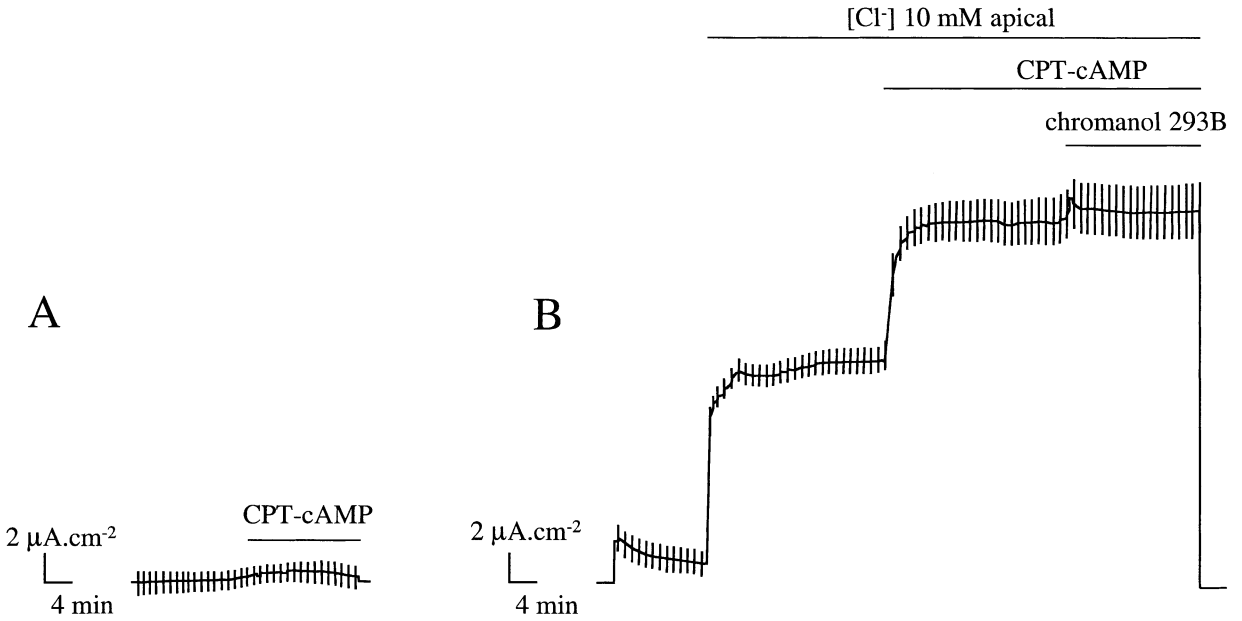

Fig. 6. Chromanol 293B does not affect the CPT-cAMP-stimulated I_{sc}. CPT-cAMP (300 μM) was added to the basolateral side of the Ussing chamber in the absence (*A*) or in the presence (*B*) of a transepithelial chloride gradient. The cAMP-stimulated I_{sc} is observed only in the presence of a Cl^- gradient and is insensitive to 100 μM chromanol 293B application (both sides).

on both sides of the cells (31.7 ± 2.0 μA cm^{-2} and 29.0 ± 1.1 μA cm^{-2}, $n = 4$ with and without chromanol application). Nor was the CPT-cAMP-stimulated I_{sc} affected by 30 μM clofilium applied on both sides of the cells (26.3 ± 1.8 μA cm^{-2} and 25.2 ± 1.9 μA cm^{-2}, $n = 5$ with and without clofilium application). This finding points to the lack of involvement of KCNQ1 channels in the cAMP-dependent Cl^- secretion in 16HBE14o− cells.

Next, we investigated the presence of K_{cAMP} channels in 16HBE14o− cells by measuring ^{86}Rb effluxes through the basolateral membranes. Permeable CPT-cAMP application (300 μM) did not affect the ^{86}Rb effluxes through the apical (*not shown*) or basolateral membranes (Fig. 7*A*). In addition, treatment of the cells with permeant cAMP did not change the ionomycin-induced ^{86}Rb efflux (Fig. 7*B*). Furthermore, 100 μM chromanol 293B, a specific inhibitor of the KCNQ1 channel (Gerlach et al., 2001; Kerst et al., 2001), had no effect on the resting or ionomycin-induced ^{86}Rb effluxes (*data not shown*). These findings also point to the absence of functional cAMP-sensitive K^+ channels in 16HBE14o− cells.

Analysis of SK and KCNQ1 Channel Expression in 16HBE14o− Cells by RT-PCR

Using specific primers for the four different SK family members and for the KCNQ1 channel, expression of the mRNA of these channels was analyzed in 16HBE14o− cells by RT-PCR. As reported in Fig. 8*A*, specific amplification products of the expected size were detected only for SK1 and SK4 channels and we failed to detect SK2 and SK3 mRNA. In addition, the KCNQ1 mRNA was also detected (Fig. 8*B*). Comparison of the sequenced PCR products with SK1, SK4 and KCNQ1 published sequences (National Center for Biotechnology Information) confirmed the mRNA presence of these channels in 16HBE14o− cells. As positive controls (Fig. 8*C*) for SK2 and SK3 primers, we amplified the corresponding mRNA from Jurkat T cells or TE671 cells, since their expression was reported by Jager, Adelman & Grissmer (2000) and by Carignani et al. (2002) respectively.

Discussion

Calcium-induced Cl^- Secretion

In the present study ionomycin was used to elicit the calcium-induced Cl^- secretion in 16HBE14o− cells. A biphasic profile comprising a transient I_{peak} and a sustained $I_{plateau}$ was observed, suggesting the presence of distinct calcium-dependent Cl^- conductances. This interpretation is mainly based on the differences in the pharmacological profile of the two I_{sc} phases. Glybenclamide, NFA and NPPB presented inhibitory effects on I_{peak} and $I_{plateau}$, but the sensitivities of both phases were found to be different (*see* Fig. 1). Also, DIDS partially blocked I_{peak}, but had no effect on $I_{plateau}$. Recently, in human bronchial epithelial cells, an interleukin-13-enhanced calcium-activated

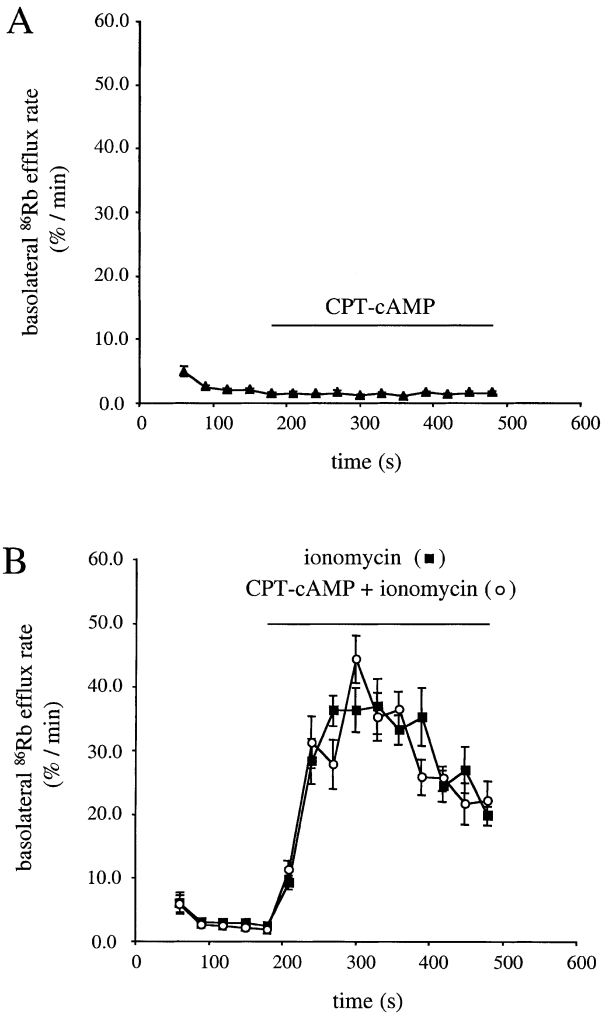

Fig. 7. CPT-cAMP does not stimulate basolateral resting and ionomycin-stimulated ^{86}Rb efflux rates. CPT-cAMP (300 μM) was added in the basolateral bathing medium without (*A*) or with (*B*) 0.2 μM ionomycin.

Cl^- conductance was also inhibited by DIDS and NPPB (Atherton et al., 2003). It has been reported that the inhibition of Ca^{2+}-activated Cl^- current by DIDS is voltage dependent, the largest effect being found for relatively high depolarizing potentials while poor (or no) effects are observed at physiological potentials (Nilius et al., 1997; Qu & Hartzell, 2001). In our ionomycin experiments, the transepithelial potential (referenced to the apical side) increased from 0.6 ± 0.1 mV to 21.5 ± 0.9 mV (corresponding to I_{peak}) and then decreased to 1.4 ± 0.2 mV (corresponding to $I_{plateau}$, $n = 9$). The apical membrane potential measured in whole-cell patch-clamp experiments was found to be around −36 mV under resting conditions (Koslowsky et al., 1994; our unpublished results). Therefore, the apical membrane potential following ionomycin application is expected to hyperpolarize during the peak phase and then depolarize during the plateau phase. The inhibitory effect of DIDS should then be more pronounced for the plateau phase than for the peak phase. This was not observed in our experiments, indicating a difference in sensitivity to DIDS of I_{peak} and $I_{plateau}$, which is not related to the voltage dependence of this inhibitor, and further supports the hypothesis of the existence of two distinct Cl^- conductances activated by the Ca_i increase.

K^+ channels have been attributed to play a key role in the establishment of the driving force for apical Cl^- exit. In several airway epithelia, the opening of basolateral K_{Ca} channels (after a Ca_i^{2+} increase) hyperpolarizes the membrane potential, leading to apical Cl^- exit through open channels (Welsh & McCann, 1985; Welsh & Liedtke, 1986; Koslowsky et al., 1994; Devor et al., 1999). In 16HBE14o− cells, K_{Ca} channel activation was found to be associated with the calcium-induced I_{sc} increase because clotrimazole or TPA application blocked the I_{sc} response (I_{peak} and $I_{plateau}$). These data show that in this cell line Ca^{2+}-mediated chloride secretion is dependent on the activation of K_{Ca} channels. However, we also show that a calcium-induced I_{sc} stimulation can still be observed in the presence of K_{Ca} blockers when a Cl^- gradient favoring Cl^- exit is generated by lowering apical Cl^- concentration. This finding suggests that calcium has an effect on the apical Cl^- conductance distinct from that on K_{Ca} conductance, i.e., the apical Cl^- conductance(s) is modulated either directly by calcium or indirectly by a calcium-dependent signaling pathway. An outwardly rectifying Cl^- channel stimulated by 1–10 μM Ca_i^{2+} concentrations has been identified in 16HBE14o− cells, in the inside-out membrane-patch configuration (Jeulin et al., 2000). In the whole-cell configuration, an ionomycin-stimulated Cl^- conductance could also be observed (our unpublished data). Therefore, calcium-activated Cl^- channels are present in 16HBE14o− cells and their activity may account for the I_{peak} and/or $I_{plateau}$ phases. Considering the pharmacology of the plateau phase (large sensitivity to glybenclamide and insensitivity to DIDS) and that of the peak phase (moderate sensitivity to glybenclamide and DIDS), we examined whether a fraction of the calcium-activated current was mediated by CFTR. Our attempts to inhibit the calcium-activated current, by treating 16HBE14o− cells with H-89, a PKA inhibitor, failed. However, a direct activation of CFTR by PKC has also been described (Tabcharani et al., 1991; Dechecchi et al., 1993; Paradiso et al., 2001). Treatment of the 16HBE14o− cells with chelerytrine, a specific PKC inhibitor, induced a small (18%) inhibitory effect on the calcium-activated current, indicating that the cell calcium increase may have stimulated the PKC pathway. Therefore, we cannot exclude that a fraction of the calcium-activated Cl^- current is mediated by CFTR, being more

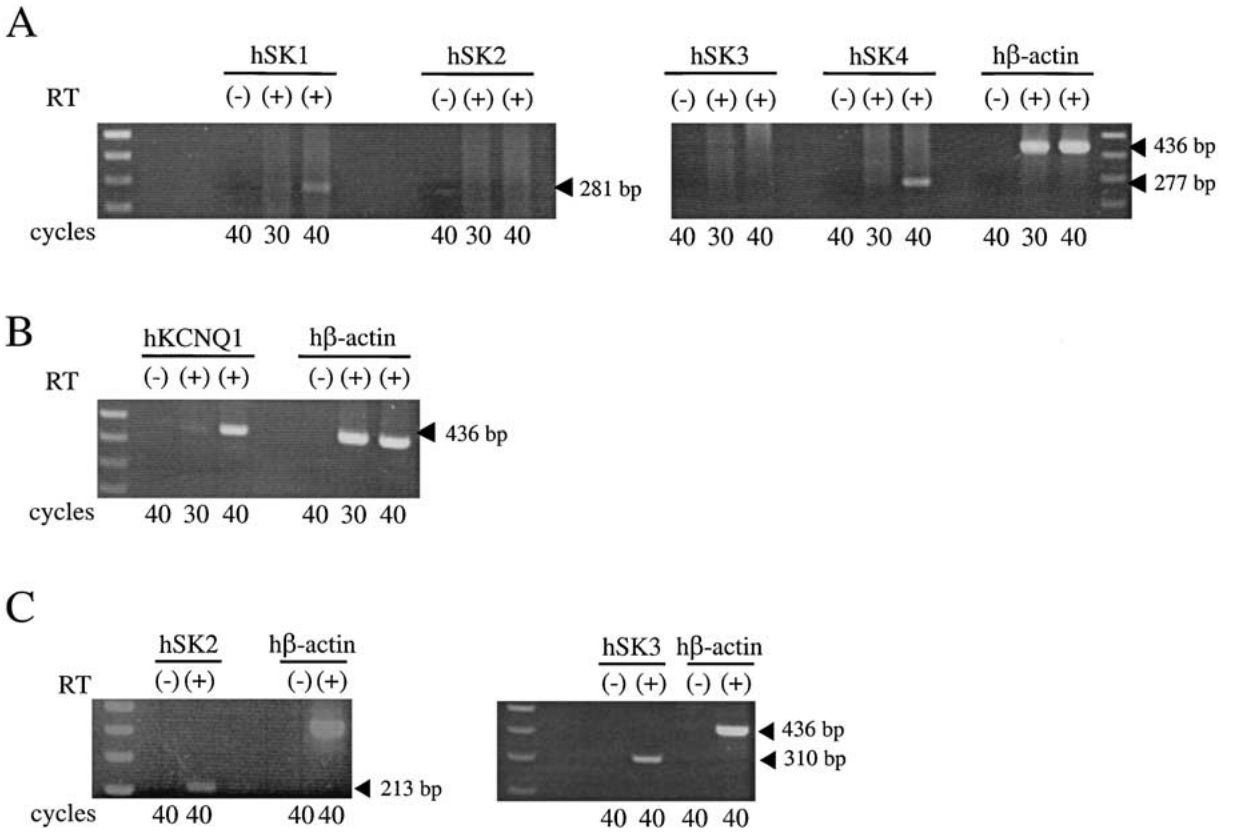

Fig. 8. Detection of SK1, SK4 and KCNQ1 mRNAs by RT-PCR in 16HBE14o−. Amplified PCR products generated using gene-specific primers (*see* Table 1) were separated on 1.7% agarose gel and size markers were used to indicate the size of the amplified fragments. Markers (200–500 bp) are indicated on the first or last lane of the gel. Without reverse transcriptase (−); with reverse transcriptase (+). β-Actin was used as a control of the RT-PCR reaction. (*A*) Detection of SK1- and SK4-, but absence of SK2- and SK3-transcripts in 16HBE14o− cells. (*B*) Detection of KCNQ1 transcripts in 16HBE14o− cells. Subsequent sequencing of the amplified fragments confirmed amplification of human SK1, SK4 and KCNQ1 in these cells. (*C*) Positive controls of SK2 and SK3 expression in cultured Jurkat T cells and in TE671 cells, respectively.

easily detected during the largest electrochemical gradient phase (i.e., during the I_{peak} phase).

Identity of Calcium-activated Chloride Channels

Two families of Ca^{2+}-activated Cl^- channels have been identified to date. The CLCA family was first described by Cunningham et al. (1995) and the molecular and functional characteristics of several of its members have been reviewed by Fuller & Benos (2000) and by Pauli et al. (2000). Among them, hCLCA1 was suggested as a mediator of calcium-activated Cl^- conductance in human intestine and was proposed as a potential candidate for an alternative pathway for Cl^- secretion in cystic fibrosis (Gruber et al., 1998). This protein was also found to be expressed in human primary lung cultures treated by interleukin-9 and has been potentially involved in asthma (Nakanishi et al., 2001; Zhou et al., 2001). Another CLCA member, hCLCA2, identified in human mammary gland, trachea and lung (at mRNA levels) has also been suggested as an alternative chloride channel in cystic fibrosis (Gruber et al., 1999).

Petrukhin and co-workers (1998) identified the VMD2 gene mutated in Best disease, encoding proteins named bestrophins. Recently, several bestrophin family members have been identified in human tissues (Marmorstein et al., 2000; Stöhr et al., 2002; Sun et al., 2002) and were localized on basolateral membranes of the retinal pigment epithelium (Marmorstein et al., 2000). One member of the bestrophin family, hBest1, elicited a calcium-sensitive Cl^- conductance inhibited by DIDS when expressed in HEK-293 cells (Sun et al., 2002). These authors came to the conclusion of a possible involvement in Cl^- secretion of this putative Cl^- channel in the retinal pigment epithelium.

We have identified several members of the CLCA and bestrophin families in 16HBE14o− cells by RT-PCR (our unpublished data). However, additional approaches are necessary to reveal the molecular identity of the calcium-stimulated Cl^- conductance(s) found in 16HBE14o− cells.

Pharmacological Characterization of K_{Ca} Channels in the 16HBE14o− Cell Line

We found that ^{86}Rb effluxes were increased by ionomycin or ATP application, indicating the presence of a calcium-sensitive K^+ permeability in these cells. Three classes of calcium-activated K^+ channels, large (BK), intermediate (IK) and small (SK) conductance calcium-activated K^+ channels (for review *see* Latorre et al., 1989), have been described and can be distinguished by their distinct pharmacological profiles. Apamin, a bee-venom peptide is a potent inhibitor of native K^+ channels (Vincent, Schweitz & Lazdunsky, 1975; Blatz & Magleby, 1986) and of SK2 and SK3 channels (Kohler et al., 1996; Bond, Maylie & Adelman, 1999), whereas the sensitivity of SK1 channel to apamin is controversial and may depend on the expression system (Shah & Haylett, 2000; Dale et al., 2002). Iberiotoxin and charybdotoxin are two scorpion-venom peptides but while the former is effective only on BK channels, the latter also inhibits the intermediate-conductance calcium-activated K^+ channels (IK) (Garcia et al., 1991). IK1/SK4 is the fourth cloned member of the SK channel family (Ishii et al., 1997; Joiner et al., 1997). In transfected CHO cells, SK4 was found to be blocked with high affinity by charybdotoxin (Joiner et al., 1997) and by clotrimazole when expressed in *Xenopus* oocytes (Ishii et al., 1997; Warth et al., 1999). Conversely, SK4 was not blocked by apamin and iberiotoxin (Ishii et al., 1997; Joiner et al., 1997). In addition, clotrimazole was found to inhibit SK4 channels with an $IC_{50} < 0.1$ μM (Ishii et al., 1997; Warth et al., 1999), while larger concentrations ($IC_{50} > 20$ μM; Wulff et al., 2000) were necessary to block SK2 or SK3 channels.

In 16HBE14o− cells, calcium-stimulated ^{86}Rb effluxes through basolateral membranes were 3.5 to 5 times larger than those through the apical membranes. They were inhibited by TPA, charybdotoxin and clotrimazole but unaffected by iberiotoxin or apamin application. The SK-channel opener 1-EBIO also elicited ^{86}Rb effluxes that were inhibited by low concentrations of clotrimazole ($IC_{50} < 200$ nM). Taken together, these data point to the presence of SK channels, and in particular to that of SK4 channels, in this bronchial epithelium. The presence of SK4 mRNA in 16HBE14o− cells is consistent with this proposal. Furthermore, our data are in agreement with a previous report in which SK4 was suggested to mediate the K_{Ca} current in native airway epithelia (Devor et al., 2000; Mall et al., 2003) as in the Calu-3 cell line (Devor et al., 1999; Cowley & Linsdell, 2002).

We described the existence of a significant Ca^{2+}-dependent apical K^+ permeability. Apical ^{86}Rb effluxes were blocked by TPA, clotrimazole and charybdotoxin and were also elicited by 1-EBIO, suggesting that SK4-like channels are likely candidates for K_{Ca} channels at the apical membrane of 16HBE14o− cells. The presence of K^+ channels located at the apical membrane of polarized cells has also been described in renal epithelia (Schwiebert et al., 1999), rat distal colon (Butterfield et al., 1997) and human airways (Frizzell, 1999; Devor et al., 2000). The relative contribution of these apical K_{Ca} channels to the electrochemical gradient appeared rather minor compared to that of basolateral K_{Ca} channels. Indeed, blockers of SK4 channels applied on the apical side of the 16HBE14o− epithelium did not affect the calcium-induced Cl^- secretion, whereas when applied on the basolateral side, they did (Fig. 5). It can, however, be remarked that apical application of charybdotoxin or apamin induces a transient increase of $I_{plateau}$, which may be due to the closure of apical K_{Ca} channels. Thus, we cannot exclude that the opening of K_{Ca} channels may lead to a small underestimation of the dominant calcium-activated Cl^- current. The physiological role of these apical K^+ channels remains to be determined. One possibility is that upon agonist stimulation, local calcium changes limited to the apical membranes affect the apical membrane potential via apical K_{Ca} channels and thus favor Cl^- secretion. In our ionomycin or ATP experiments, these effects could be masked by the large Ca_i^{2+} changes, which stimulate the dominant basolateral K_{Ca} channels.

If SK4 is a candidate for apical K_{Ca} channels in 16HBE14o− cells, the slight but significant sensitivity of apical ^{86}Rb effluxes to apamin may also suggest the presence of other SK members. The presence of SK1 at the apical membrane is a possibility, since we also detected SK1 mRNA, but not those of the SK2 and SK3 channels.

Are K_{cAMP} Channels Involved in Cl^- Secretion in 16HBE14o− Cells?

KCNQ1 is a small-conductance K^+ channel widely distributed in epithelial (Takumi, Ohkubo & Nakanishi, 1988; Neyroud et al., 1997; Köttgen et al., 1999) and non-epithelial tissues (Yang et al., 1997). KCNQ1 channels expressed in *Xenopus* oocytes were found to be stimulated by cAMP, calcium and alkaline pH (Kerst et al., 2001) and inhibited by chromanol 293B and clofilium (Gerlach et al., 2001; Kerst et al., 2001; Honoré et al., 1991). It has been proposed that the KCNQ1/KCNE3 complex assembles to form the K^+ channel that is involved in cAMP-stimulated intestinal Cl^- secretion occurring in secretory diarrhea and in cystic fibrosis (Schroeder et al., 2000). In Calu-3 cells, KCNQ1 and two of its subunits KCNE2 and KCNE3 were reported and the authors suggested a role of KCNQ1/KCNE3 in Cl^-

secretion in this cell line (Cowley & Linsdell, 2002). In addition, in native nasal cells, Mall et al. (2003), recently presented evidence of the implication of a chromanol 293B-sensitive K^+ conductance on the UTP-stimulated I_{sc}.

To investigate the possible involvement of K_{cAMP} in the Cl^- secretion in 16HBE14o− cells we determined the effect of an increase of intracellular cAMP on ^{86}Rb effluxes and I_{sc}. Treatment of the cell monolayer with CPT-cAMP (Fig. 7) or with forskolin and IBMX (unpublished data) failed to stimulate apical and basolateral ^{86}Rb effluxes. In addition, the CPT-cAMP-stimulated I_{sc} was not inhibited by the K_{cAMP} blockers chromanol 293B or clofilium. Therefore, we did not observe functional K_{cAMP} channels in 16HBE14o− cells upon cAMP stimulation. This finding differs from that of Mall et al. (2000) who reported that a small fraction (about 18%) of the whole-cell conductance was inhibited by chromanol 293B after stimulation of 16HBE14o− cells by forskolin. Nevertheless, we confirmed the occurrence of KCNQ1 mRNA in 16HBE14o− cells, as reported by Mall et al. (2000). The reason for this discrepancy may be due to different experimental approaches and/or to the state of differentiation of 16HBE14o− cells because we used polarized monolayers. Since the properties of the KCNQ1 channel depend on its association with KCNE β-subunits (Barhanin et al., 1996; Busch et al., 1997; Schroeder et al., 2000), a possibility would be the absence in 16HBE14o− cells of a key KCNE β-subunit modulating the activity of cAMP-dependent K^+ channels.

In conclusion, two distinct components (I_{peak} and $I_{plateau}$) of Cl^- secretion were identified by a pharmacological approach in 16HBE14o− cells, after Ca_i^{2+} increase. However, further experiments are necessary to identify the Cl^- channels underlying these calcium-activated currents. K_{Ca} channels presenting the pharmacology of SK4 channels were present on apical and basolateral membranes of the epithelial cells, with a major role of the basolateral SK4-like channels in calcium-activated Cl^- secretion. SK1 channels could account for the small apamin sensitivity found for the apical K^+ permeability. Considering the key role of basolateral SK4-like channels in Cl^- secretion, the determination of factors involved in their regulation could represent therapeutic targets in the treatment of cystic fibrosis and should therefore be explored.

We thank Dr. D.C. Gruenert for kindly providing the 16HBE14o− cells and Dr. P. Durbec for the TE671 cell line and Dr. B. Rossi for Jurkat T cells. This work was supported by the CNRS, the MENRT and the "Vaincre la Mucoviscidose" Association (Paris, France). We thank Dr. S. Schmieder for critical reading of the manuscript.

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