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Chloride Currents Activated by Calcitonin and cAMP in Primary Cultures of Rabbit Distal Convoluted Tubule

M. Tauc, M. Bidet, P. Poujeol
URA CNRS 1938, Université de Nice Sophia Antipolis, Parc Valrose 06108 Nice Cedex 2, France

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Abstract. Chloride (Cl^-) conductances were studied in primary cultures of the bright part of rabbit distal convoluted tubule (DCTb) by the whole cell patch clamp technique. The bath solution (33°C) contained (in mM): 140 NaCl, 1 $CaCl_2$, 10 *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4 and the pipette solution 140 *N*-methyl-D-glucamine (NMDG)-Cl, 5 MgATP, 1 ethylene-glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 HEPES, pH 7.4. We identified a Cl^- current activated by 10^{-5} M forskolin, 10^{-3} M 8-bromo adenosine 3′,5′-cyclic monophosphate (8 Br-cAMP), 10^{-6} M phorbol 12-myristate 13-acetate (PMA), 10^{-3} M intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) and 10^{-7} M calcitonin. The current-voltage relationship was linear and the relative ion selectivity was $Br^- > Cl^- \gg I^- >$ glutamate. This current was inhibited by 10^{-3} M diphenylamine-2-carboxylate (DPC) and 10^{-4} M 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and was insensitive to 10^{-3} M 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). These characteristics are similar to those described for the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- conductance. In a few cases, forskolin and calcitonin induced an outwardly rectifying Cl^- current blocked by DIDS. To determine the exact location of the Cl^- conductance 6-methoxy-1-(3-sulfonatopropyl) quinolinium (SPQ) fluorescence experiments were carried out. Cultures seeded on collagen-coated permeable filters were loaded overnight with 5 mM SPQ and the emitted fluorescence analyzed by laser-scan cytometry. Cl^- removal from the apical solution induced a Cl^- efflux which was stimulated by 10^{-5} M forskolin, 10^{-7} calcitonin and inhibited by 10^{-5} M NPPB. In 140 mM NaBr, forskolin stimulated an apical Br^- influx through the Cl^- pathway. Forskolin and calcitonin had no effect on the basolateral Cl^- permeability. Thus in DCTb cultured cells, exposure to calcitonin activates a Cl^- conductance in the apical membrane through a cAMP-dependent mechanism.

Key words: Whole cell—Chloride conductance—SPQ fluorescence—cAMP—Calcitonin—Cultured distal tubule

Introduction

Chloride (Cl^-) channels have been extensively studied in secretory epithelia. They play a key role in the secretion of fluid through the apical membrane. Of the various Cl^- channels, the most investigated is the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. As in secretory epithelia, Cl^- channels with very diverse and distinct properties have been described in the kidney. In this complex structure, any segment of the nephron from glomerulus to medullary collecting duct, may transport chloride by a mechanism different from that of other segments and this transport may be regulated through different pathways. For example, it has been demonstrated that rabbit proximal convoluted tubule (PCT) cells in culture, possess an apical outwardly rectifying Cl^- channel activated by parathyroid hormone (PTH) via the protein kinase A (PKA) and proteine kinase C (PKC) pathways (Suzuki et al., 1991). In rat PCT grown in primary culture, a more recent study describes at least four different Cl^- selective channels located in the apical membrane and modulated by cyclic nucleotides, calcium (Ca^{2+}) or voltage (Darvish, Winaver & Dagan, 1994). In mouse microdissected thick ascending limbs of Henle's loop (TAL), basolateral Cl^- channels of 40 pS conductance regulated by arginine vasopressine (AVP) via the PKA pathway have been recorded (Paulais

Correspondence to: P. Poujeol

& Teulon, 1990). In the bright part of the distal convoluted tubule (DCTb), we identified a small-conductance, adenosine 3′,5′-cyclic monophophosphate (cAMP)-activated Cl^- channel in the apical membrane of cultured rabbit DCTb (Poncet et al., 1994). This channel showed striking similarities to the CFTR Cl^- channel. A Cl^- channel with similar properties has also been recorded in the apical membrane of cultured cortical collecting tubule (CCT) (Ling & Kokko, 1992) and inner medullary collecting tubule (IMCT) (Vandorpe et al., 1993). In these tight epithelia, an interesting feature was that the Cl^- channel could be controlled by polypeptide hormones (Nagy, Naray-Fejes-Toth & Fejes-Toth, 1994) and other effectors (Rocha & Kudo, 1990; Ikeda et al., 1993) coupled to adenylate cyclase. In view of these results, we carried out whole cell clamp and fluorescence experiments to investigate further the Cl^- permeability of cultured DCTb cells and to study its regulation by calcitonin. We demonstrated that only the apical membrane of DCTb cells exhibited a Cl^- permeability stimulated by calcitonin via the PKA/cAMP pathway. Two different conductances underline the stimulated Cl^- permeability: a linear CFTR-like Cl^- current and an infrequent outwardly rectifying Cl^- current. A basolateral Cl^- permeability also occurs but the regulation of this remains unknown.

Material and Methods

Primary Cultures

The primary cell culture technique has been described in detail in previous papers (Tauc et al., 1989; Mérot et al., 1989). The bright part of the rabbit distal tubule was microdissected under sterile conditions from 4–5 week-old male New Zealand rabbit kidneys. The kidneys were perfused with Hank's solution (Gibco) containing 600–700 U/ml collagenase (Worthington) and were then cut into small pyramids which were incubated in medium containing 150 U/ml collagenase. The tubules were seeded in collagen-coated 35 mm Petri dishes or in collagen coated polycarbonate filters filled with a culture medium composed of equal quantities of Dubelcco Modified Eagle Medium (DMEM) and Ham F12 (Gibco), containing 15 mM $NaHCO_3$, 20 mM *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 7.5, 2 mM glutamin, 5 μg/ml insulin, 50 nM dexamethasone, 10 ng/ml epidermal growth factor, 5 μg/ml transferrin, 30 nM sodium selenite, 10 nM triiodothyronine. Cultures were maintained at 37°C in a 5% CO_2/95% air-water-saturated atmosphere. The medium was changed 4 days after seeding and then every two days.

Whole Cell Experiments

Experimental Protocol

Whole cell currents were recorded from 12–22 day-old cultured cells grown on collagen-coated supports maintained at 33°C throughout experiments. The ruptured-patch whole cell configuration of the patch clamp technique was used. Patch pipettes were made from borosilicate capillary tubes (1.5mm OD, 1.1mm ID, Clay Adams) using a two-stage vertical puller (PP 83, Narishige, Tokyo) and filled with *N*-methyl-D-glucamine chloride (NMDGCl) solution, had a resistance ranging from 2 to 3 MΩ in NaCl buffer. To reduce junction potentials, the reference electrode was an Ag/AgCl pellet bathed in the same solution as that in the pipette, and connected to the bath via a 3 M KCl agar bridge. Cells were observed with an inverted microscope (Zeiss IM 35) the stage of which was equipped with a water robot micromanipulator (WR 89, Narishige). The patch pipette was connected via an Ag/AgCl wire to the headstage of a RK 300 patch amplifier (Biologic). After the formation of a giga-ohm seal the fast compensation system of the amplifier was used to compensate for the head-stage intrinsic input capacitance and the pipette capacitance. The membrane was ruptured by additional suction to achieve the conventional whole cell configuration. At this stage, the cell capacitance (Cm) was compensated by using the corresponding knob setting on the RK 300 amplifier. In 292 experiments, the Cm averaged 16.5 ± 0.51 pF. The series resistances (*Rs*) were calculated from the equation $Rs = \tau/Cm$ where τ is the time constant of the exponential relaxation. In 292 experiments, *Rs* averaged 12.3 ± 0.35 MΩ. No series resistance compensation was applied but experiments in which the series resistance was higher than 20 MΩ were discarded. Cell membrane potentials (V_m) were measured at zero membrane current in the I_o mode of the amplifier. To check for electrical coupling between cells, some whole cell experiments were performed on trypsinized cells, for which cultured DCTb cells were treated for 5 min with a Ca^{2+} and Mg^{2+}-free Hank's solution containing 5 mg/ml trypsin and 0.5 mM EDTA. Cells were rinsed in NaCl solution and isolated cells were patched. Solutions were perfused in the extracellular bath by using a four-channel glass pipette, the tip of which was placed as near as possible to the clamped cell. Before perfusion of calcitonin, the monolayer was carefully striated around the clamped cell in order to improve the access of the hormone to the basolateral membrane. To prevent the occurrence of swelling activated Cl^- currents due to the hydrostatic pressure of the pipette, most experiments were performed in extracellular solutions made hyperosmotic (350 mosm/l) by addition of 60 mM mannitol (Worrel et al., 1989).

Data Acquisition and Analysis

Voltage clamp commands, data acquisition and data analysis were controlled by an IBM AT-compatible computer equipped with a Digi data 1200 interface (Axon Instruments, Foster City, CA). pClamp software (versions 5.51 and 6.0 Axon Instruments) was used to generate whole cell *I-V* relationships. The membrane current resulting from voltage stimuli was filtered at 1 kHz, sampled at a rate of 2560/sec and stored directly on the hard disk. Cells were held at a holding potential (Vhold) of −50 mV and 400 msec pulses from −100 to +120 mV were applied with increments of 20 mV every 2 sec.

Solutions

The compositions of the various solutions are given in Table 1.

Fluorescence Experiments

Fifteen to twenty day-old DCTb cultures grown on collagen-coated filters were loaded for 12–16 hr at 37°C, with 5 mM 6-methoxy-1-(3-sulfonatopropyl) quinolinium (SPQ) added to the culture medium. Confluent cultures growing on filters were carefully rinsed with NaCl solution (in mM): NaCl 140, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1, glucose 5, HEPES 20, pH 7.4 and mounted in an Ussing chamber (aperture 7mm^2) with the apical face directed downwards. This chamber was then

Table 1. Composition of solutions used in whole cell clamp experiments

	NaCl sol.	NaI sol.	NaBr sol.	NMDGCl sol.	Glutamate sol.	Pipette sol.
Na^+	140	140	140		140	
Ca^{++}	1	1	1	1	1	
$NMDG^+$				140		140
Cl^-	142	2	2	142	2	140
I^-		140				
Br^-			140			
Glutamate					140	
EGTA						1
ATP						5
Glucose	5	5	5	5	5	
HEPES	10	10	10	10	10	10

Bath solutions were titrated to pH 7.4 with NaOH. Pipette solution was titrated to pH 7.4 with Tris. Solutions were prewarmed at 37°C. Concentrations are given in mM.

placed in a perfusion chamber installed on an inverted microscope stage. The perfusion chamber allowed for the independent perfusion of the apical and the basolateral membranes of the culture (Bidet et al., 1990).

Quantitative measurements of SPQ fluorescence were made with the interactive laser cytometer ACAS 570 (Meridian Instruments, Okemos, Michigan). The optical system was composed of an Olympus inverted microscope IMT2 and a Zeiss 40× objective (Ph2 LD-Plan 40) was used for epifluorescence measurement. Excitation was with a 5 W argon ion laser which produces illumination in several discrete lines over the 457.9–528.7 nm range and one in the UV spectra (351–364 nm range), the latter being used for the SPQ experiments. An acousto-optic modulator (AOM) rapidly changed the intensity and duration of the laser spot for the sequential exposure of small areas of a cell to brief laser pulses. Light emitted by the laser head entered the optical crystal of the AOM. In operation, a radio frequency acoustical wave is generated within the crystal; by varying the control voltage to the AOM driver module, the computer regulated the amplitude of the applied radio frequency signal and thus the intensity of the sample illuminating beam. In our experiments, the laser power was adjusted to 100 mW with only 10% deflected to the cells through the AOM. This device limits the exposure of biological preparations to the excitation beam by reducing the UV quantity necessary to obtain sufficient emission signals, thus preventing an excessive photobleaching of the chloride probe. The excitation laser beam (≈0.6 μm diameter) was applied to the cell monolayer through the epifluorescence port of the microscope and a UV filter block mounted in the dichroic cube (350 nm band pass excitation filter, a 380 nm dichroic mirror and a 390 nm barrier filter). Images were collected as single frames repeated every 30 sec and stored on a hard disk. After a series of frames were taken, fluorescent levels were analyzed with the image processing system. The grey-level variations from one frame to another were analyzed in different zones automatically redrawn with the Meridian software. The average of pixel grey-level intensities was calculated for each zone and the data were finally processed with LOTUS 123 software.

Cl^- influx and efflux rates were computed from the time course of intracellular fluorescence using the equation: $J_{Cl} = (F_0/K_{Cl} \,.\, F^2)(dF/dt)$ (Verkman, Chao & Hartmann, 1992) K_{Cl}: Stern-Volmer constant for quenching of intracellular SPQ by Cl^-: 9 M^{-1} (*see* calibration experiments below). *dF/dt:* initial rate of fluorescence change upon addition or removal of Cl^-. F_0*:* SPQ fluorescence in the absence of Cl^-. *F*: SPQ fluorescence at time *t.*

Cl^- efflux was induced by replacement of the NaCl solution with an iso-osmotic $NaNO_3$ solution (in mM): $NaNO_3$ 140, KNO_3 5, Ca-gluconate 3, $MgSO_4$ 1, glucose 5, HEPES 20, pH 7.4. To determine the fluorescent background, at the end of each experiment, DCTb cells were incubated in 140 mM KSCN which rapidly quenched SPQ fluorescence.

Quantitative analysis of SPQ fluorescence signals from monolayers necessitates the knowledge of the relationship between SPQ fluorescence and intracellular Cl^- activity. This fluorescence was calibrated by a double ionophore technique. Intracellular Cl^- was set equal to external Cl^- using nigericin and tributyltin, the only known compound with Cl ionophoric activity (Krapf, Berry & Verkmann, 1988). Figure 1*A* shows a typical calibration experiment. A cultured DCTb monolayer was perfused continuously with solutions containing 10 μM nigericin and 10 μM tributyltin at various Cl^- concentrations. The different Cl^- concentrations were obtained by mixing 2 buffers (buffer 1 (in mM): KCl 60, $NaNO_3$ 65, HEPES 50, $CaCl_2$ 1 pH 7.4; buffer 2: KNO_3 60, $NaNO_3$ 65, Ca-gluconate 3, HEPES 50, pH 7.4). The plot F_0/F against Cl^- concentration (Chao et al., 1989) allowed for the calculation of the Stern-Volmer constant (Fig. 1*B*). F_0/F is the total fluorescence measured in the absence of Cl^- minus that after SCN quenching divided by the fluorescence in the presence of Cl^- minus that after SCN quenching. In cultured DCTb cells K_{Cl} was equal to 9 M^{-1}.

Drugs

Forskolin (Sigma) was prepared as 10 mM stock solution in ethanol and dissolved at 10 μM in buffer solutions. 8 bromo cyclic AMP (Sigma) was directly dissolved at 1 mM in buffer solutions. 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) from Calbiochem was prepared at 100 mM in DMSO and used at 0.1 mM in final solutions. Diphenylamine-2-carboxylate (DPC) from Aldrich was prepared as 1 M stock solution in DMSO and dissolved at 1 mM in incubation medium. 4-4′-diisothiocyanostilbene-2,2′ disulfonic acid (DIDS) from Sigma was directly dissolved at a final concentration of 1 mM. Human calcitonin (Cibacalcin 0.5 mg) was from Ciba-Geigy (France). All other products were from Sigma.

Results

Effects of cAMP and Calcitonin on Cl^- Currents in Cultured Distal Cells

Linear Currents

Whole cell currents were recorded with pipette Ca^{2+}-free solutions containing NMDGCl as the major cation and extracellular solutions containing NaCl. After successful giga-ohm seal formation, the whole cell configuration was obtained in 25% of the cases. Just after the rupture of the cell membrane, the membrane potential (measured in current clamp mode) averaged −25.9 ± 2.2 mV (n = 49), after which within 2–4 min the cell depolarized as the pipette solution equilibrated with the cell interior. Voltage-clamp experiments were performed holding the cell at −50 mV and applying, voltage steps of 400 msec duration every 2 sec from −100 to 120 mV in 20 mV increments. In a large majority of cells, the voltage-step

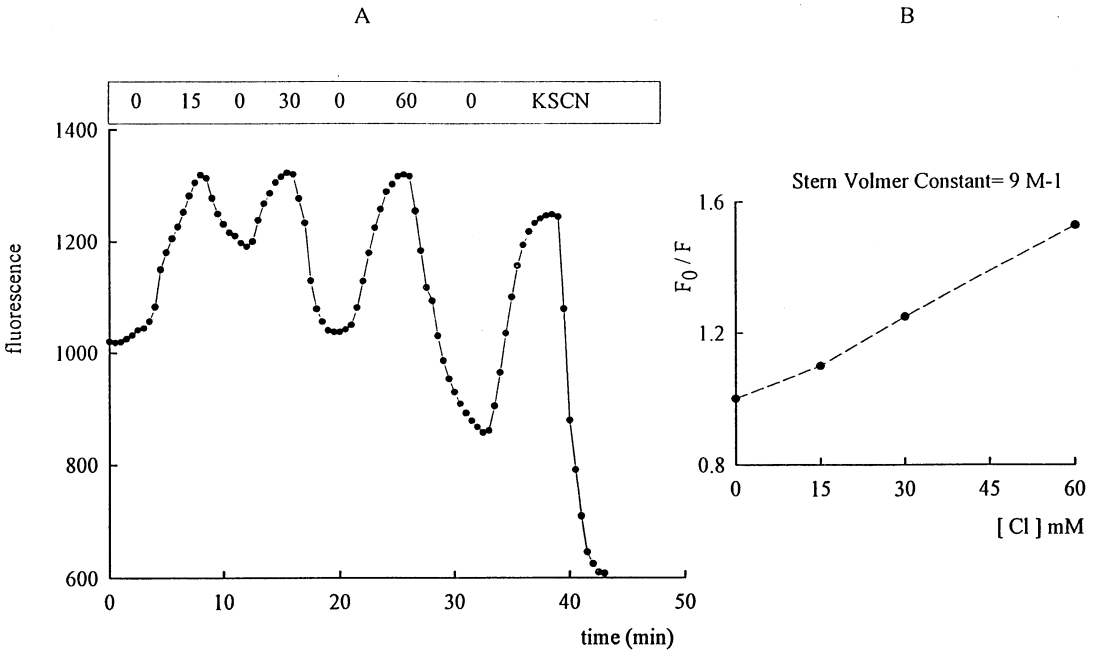

Fig. 1. Calibration experiment in SPQ-loaded DCTb cells. (*A*) The intracellular SPQ time course was detected during variations of the extracellular Cl^- concentration between 0 and 60 mM in the presence of nigericin and tributyltin. Each point corresponds to the average fluorescence of 20 to 30 cells. The background fluorescence was determined at the end of the experiment by adding 140 mM KSCN. (*B*) Calibration curve in the form of a Stern-Volmer plot. F_0/F is SPQ fluorescence measured in the absence of Cl^- divided by that in the presence of Cl^-. The slope of the curve gives a Stern-Volmer constant of 9 M^{-1}.

protocol elicited small currents (Fig. 2*A*) that changed linearly with the membrane voltage with a slope conductance of 2.14 ± 0.26 nS and a reversal potential of −1.8 ± 0.9 mV, $n = 40$ (Fig. 2*E*). The amplitude of the currents was 143 ± 14 pA at 80 mV. Because of this small amplitude, the nature of the current was not analyzed further. However, the reversal potential indicates that Cl^- may well be the charge carrier. In a previous paper (Poncet et al., 1994), we found that cAMP was implicated in the regulation of chloride channels in cultured DCTb, in view of which we evaluated the role of cAMP in the whole-cell current. Exposure to 10^{-3} M 8 bromo-cAMP or 10^{-5} M forskolin induced an increase in membrane currents (Fig. 2*B* and *C*). The maximal increase was obtained 3 to 4 min after the beginning of perfusion. Figure 2*E* shows that the activated currents presented a linear current-voltage relationship which reversed at −0.5 ± 0.3 mV, $n = 4$ and −2.0 ± 0.3 mV, $n = 22$, for 8 bromo-cAMP and forskolin respectively. The current level at +80 mV reached 1021 ± 69 pA in the presence of 8 bromo-cAMP and 853 ± 97 pA in that of forskolin. The slope conductance was 8.9 ± 1.7 nS when the current was induced by 8 bromo-cAMP and 9.4 ± 1.2 nS when stimulated by forskolin. In some experiments, 10^{-3} M cAMP was added to the pipette solution directly. This maneuver elicited linear currents (Fig. 2*D* and *E*), with maximum amplitude (362 ± 27 pA at 80 mV) 3–4 min after breaking the cell membrane. In these conditions, the cell current reversed at 0.23 ± 0.69 mV and the slope conductance was 4.4 ± 0.3 nS ($n = 13$).

The experiments yielding these data were performed in symmetrical Cl^- concentrations and in the presence of EGTA in the pipette to avoid involvement of intracellular Ca^{2+}. The reversal potential was very close to that of Cl^- and in the absence of permeable cations in the pipette, the outward current was carried by Cl^-. To eliminate any participation of cations in the inward current, some experiments were performed after replacing NaCl by NMDGCl in the bath solution. This substitution did not modify the current evoked by forskolin at each voltage step. The total current at −80 mV was not significantly different from that at +80 mV (−829.5 ± 57.9 pA *vs.* 912.8 ± 47.4, $n = 5$). In these conditions, the conductance was 9.9 ± 0.8 nS, $n = 5$.

To study the anion permeability of the cell membrane after application of forskolin, all except 2 mM of the Cl^- in the bath solution was replaced with Br^-, I^- or glutamate. Fig. 3*A–C* gives typical recordings of the currents obtained in the presence of the 3 different anions. Figure 3*D* shows current-voltage relations and Table 2 provides a summary of the reversal potentials as well as the calculated permeability ratios for a given anion. Replacing external chloride with glutamate or I^- shifted the reversal potential towards positive voltages. However, glutamate decreased the outward currents but had little effect on the inward ones whereas I^- strongly

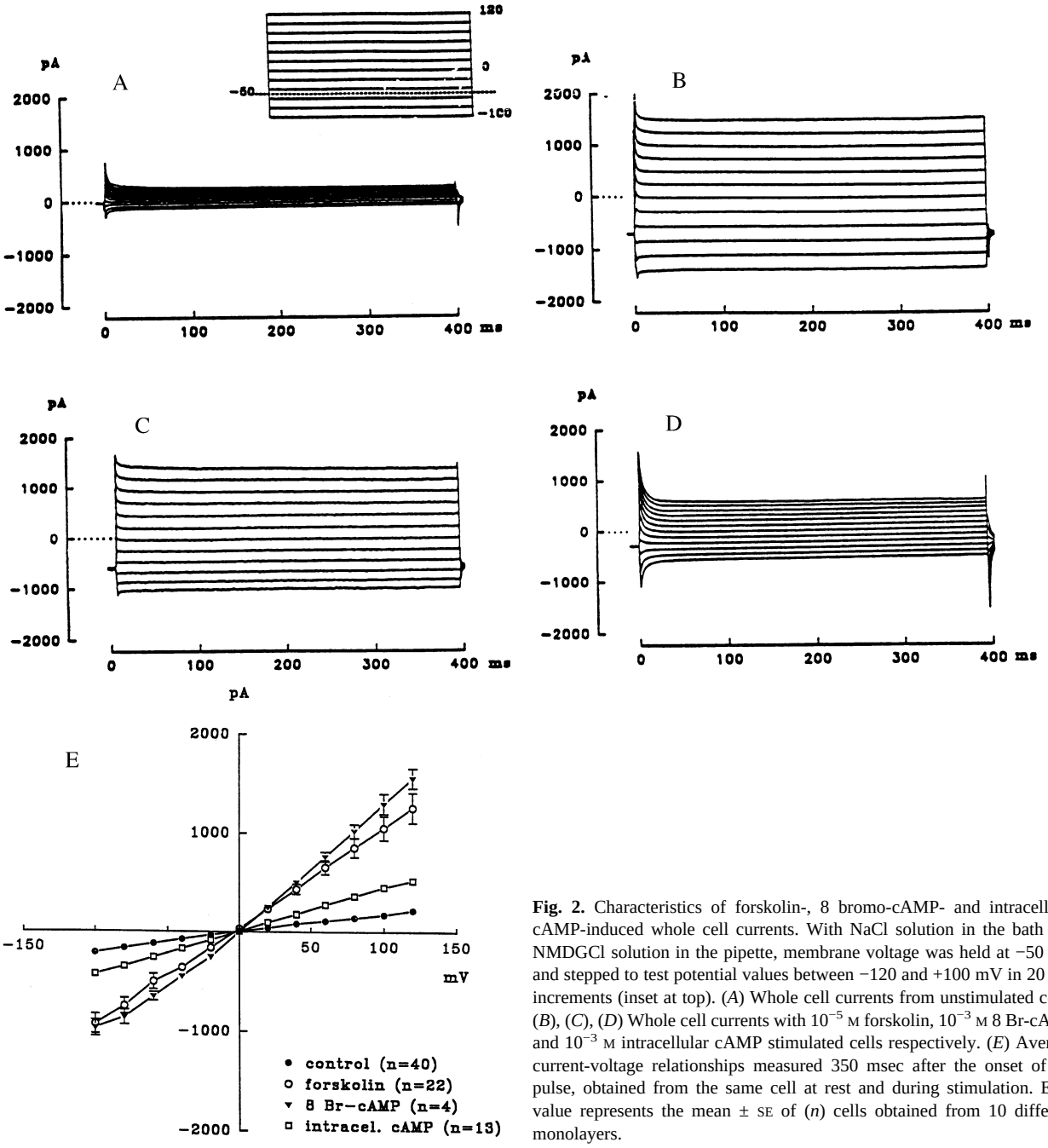

Fig. 2. Characteristics of forskolin-, 8 bromo-cAMP- and intracellular cAMP-induced whole cell currents. With NaCl solution in the bath and NMDGCl solution in the pipette, membrane voltage was held at −50 mV and stepped to test potential values between −120 and +100 mV in 20 mV increments (inset at top). (*A*) Whole cell currents from unstimulated cells. (*B*), (*C*), (*D*) Whole cell currents with 10^{-5} M forskolin, 10^{-3} M 8 Br-cAMP and 10^{-3} M intracellular cAMP stimulated cells respectively. (*E*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from the same cell at rest and during stimulation. Each value represents the mean ± SE of (*n*) cells obtained from 10 different monolayers.

blocked both outward and inward currents. In the presence of Br^-, the reversal potential shifted towards negative values. Finally, the sequence for the linear cAMP sensitive conductance was $Br^- > Cl^- \gg I^- >$ glutamate.

To characterize further the Cl^- current induced by forskolin, we tested three anion channel blockers added to the bathing solution. Figure 4*A* and *C* shows that inhibition of the whole cell Cl^- current occurred after addition of NPPB and DPC. The inhibitory effect of 10^{-4} M NPPB was 70 ± 5% ($n = 4$) and was reversible and that of 10^{-3} M DPC was 97 ± 2% ($n = 4$) (Fig. 4*D*) and irreversible. In contrast to this, forskolin-stimulated currents were not significantly modified by exposure to 10^{-3} M DIDS (Fig. 4*B*).

Calcitonin is known to stimulate adenylate cyclase in microdissected rabbit DCTd (Chabardès et al., 1976) as well as in primary cultures of DCTb (Mérot et al., 1989). As illustrated in Fig. 5, the perfusion of 10^{-7} M human calcitonin induced the linear Cl^- current after a delay of 1 min with a maximum effect at 4 min after the

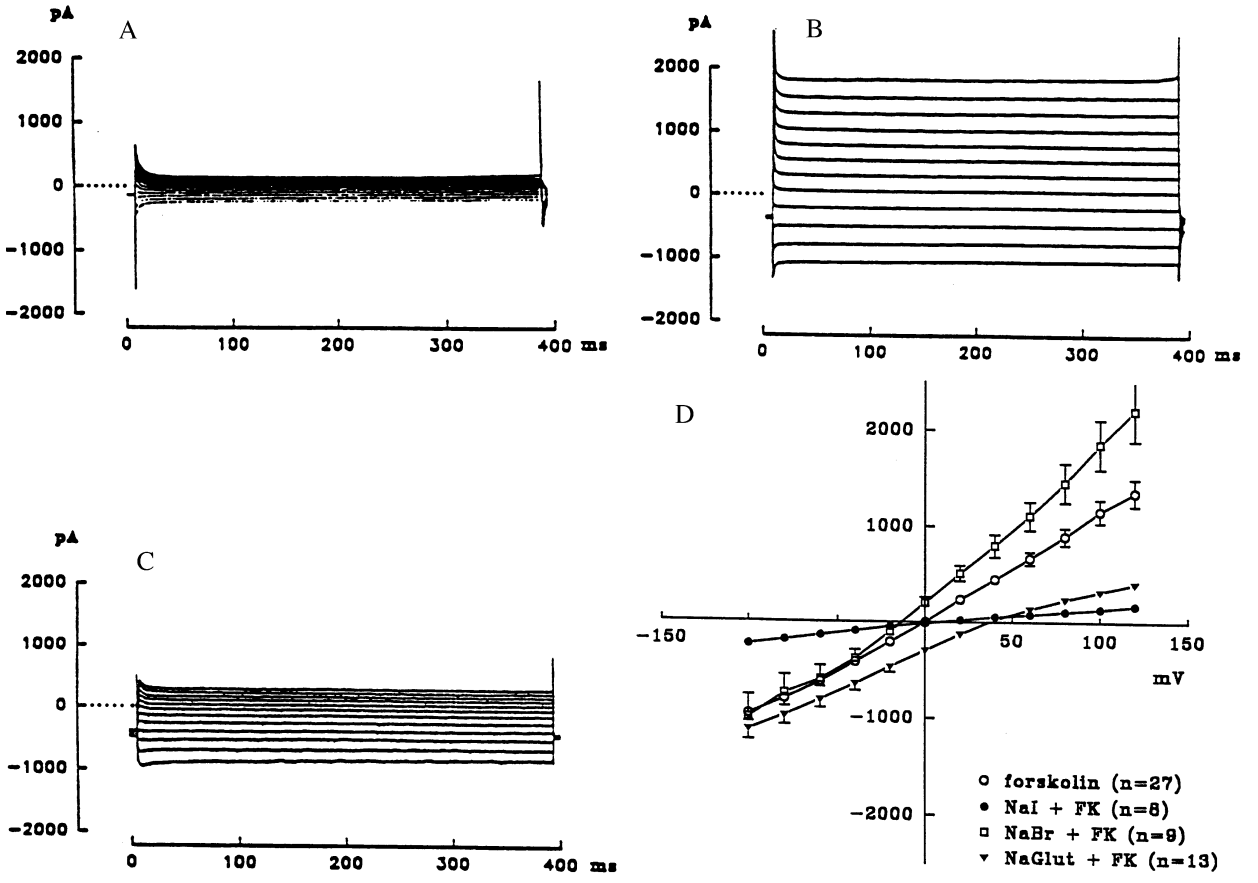

Fig. 3. Effect of extracellular chloride substitution on whole cell currents activated by forskolin. (*A*), (*B*), (*C*) Whole cell currents recorded in the presence of I^-, Br^- and glutamate respectively. (*D*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from the same cell at rest, during forskolin stimulation alone and after chloride substitution. Each value represents the mean ± SE of (*n*) cells obtained from 10 different monolayers.

beginning of perfusion. The mean conductance was 4.42 ± 0.21 nS ($n = 18$). This conductance was further investigated with halide substitution experiments. The results reported in Fig. 6 show that I^- strongly blocked, whereas Br^- increased the calcitonin-sensitive Cl^- currents. The estimated relative permeability was $Br^- > Cl^- > I^-$ (Table 2). The sensitivity to NPPB and DIDS was also tested on calcitonin-induced Cl^- currents. Exposing the cells to solutions in the presence of 10^{-4} M NPPB inhibited the activated current by 86 ± 5% ($n = 5$) (Fig. 7*C* and *E*). In contrast to this, the application of 10^{-3} M DIDS did not inhibit the activated current (Fig. 7*D* and *E*).

The effect of calcitonin was concentration dependent. Figure 8*A* shows the *I-V* relationships obtained with 4 different doses of calcitonin and Fig. 8*B* gives the dose-response curve. The current was measured by clamping the voltage at 100 mV and the calcitonin-sensitive current was calculated by substracting basal current from calcitonin-induced current recorded at 4 min. The half maximal effect calculated from this curve, occurred at 4×10^{-8} M calcitonin and the maximal effect at concentrations higher than 10^{-6} M. This concentration increased the slope conductance from 1.30 ± 0.16 nS ($n = 5$) in control conditions to 4.72 ± 0.31 ($n = 5$).

To check that activation of protein kinase C (PKC) modified the Cl^- conductance, we tested the effect of (phorbol) PMA. Preliminary results are illustrated in Fig. 9*A–C*. Application of 10^{-6} M PMA induced Cl^- currents in 9 of 19 attempts. With 140 mM NMDG Cl in the pipette and 140 mM NaCl in the bath, the current was time independent, reversed at 2.4 ± 2.1 mV ($n = 9$), and the current-voltage relationship was linear. The slope conductance was significantly increased in the presence of phorbol ester (control: 1.47 ± 0.18 nS; PMA: 4.61 ± 0.95 nS, $n = 9$, $P < 0.01$).

Nonlinear Currents

In a few cases, the currents stimulated by 10^{-5} M forskolin and 10^{-7} M calcitonin showed an outwardly rectifying relationship (Fig. 10*B, C* and *E*). In forskolin experiments, the current reversed at −2.0 ± 1.5 mV and the total

Table 2. Effect of substitution of extracellular Cl^- by various anions on E_{rev} in the presence of forskolin and calcitonin

	Forskolin		Calcitonin	
	E_{rev} (mV)	P_x/P_{Cl}	E_{rev} (mV)	P_x/P_{Cl}
Br^-	−12.4 ± 0.99 (n = 9)	1.64 ± 0.073 (n = 9)	−11.29 ± 2.00 (n = 7)	1.57 ± 0.10 (n = 7)
I^-	14.5 ± 2.8 (n = 8)	0.59 ± 0.07 (n = 8)	7.6 ± 1.7 (n = 8)	0.77 ± 0.07 (n = 8)
Glutamate	33.4 ± 1.3 (n = 13)	0.27 ± 0.01 (n = 13)		

Values are mean ± SE of (n) cells. E_{rev} for each anion is significantly different from that of other anions ($P < 0.01$, Student t test). The estimated relative permeability P_x/P_{Cl} was calculated with the equation: $E_{rev} = -58 \log ((([Cl^-]_0.P_{cl} + [X]_0.P_x)/([Cl^-]_i.P_{Cl} + [X]_i.P_x))$.

current at 100 mV was 1.6 ± 0.1 times the current at −100 mV (100 mV: 627 ± 77 pA; −100 mV: −392 ± 70 pA, n = 5, $P < 0.0001$). In the presence of calcitonin, the reversal potential was 0.8 ± 0.4 mV and the total current at 100 mV was 1.5 ± 0.6 times the current at −100 mV (100 mV: 310 ± 41 pA; −100 mV: −198 ± 26 pA, n = 6, $P < 0.002$). It is interesting that the outward rectifying current induced by forskolin was strongly

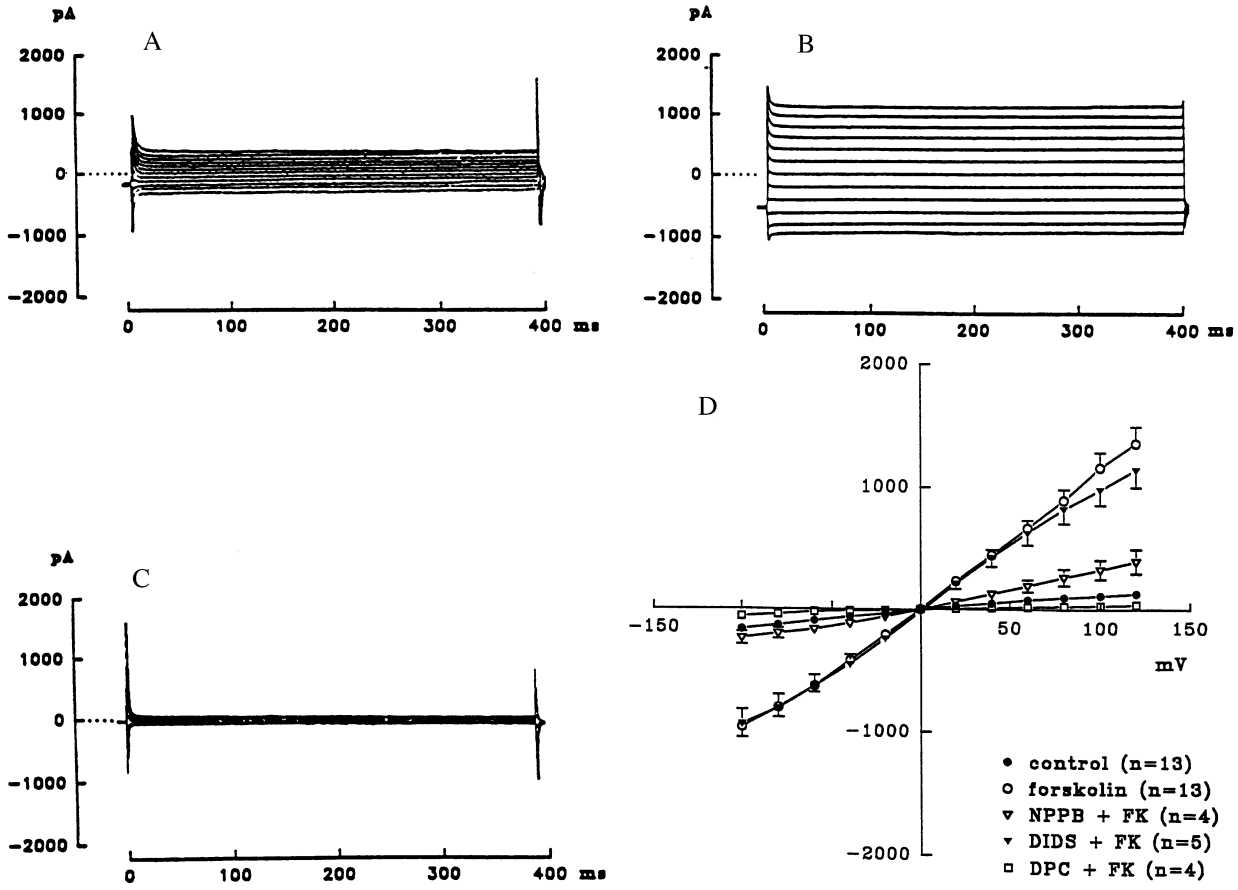

Fig. 4. Effect of chloride channel inhibitors on forskolin-activated whole cell currents. (*A*) Whole cell currents recorded during extracellular perfusion of 10^{-4} M NPPB. (*B*) With extracellular perfusion of 10^{-3} M DIDS. (*C*) With extracellular perfusion of 10^{-3} M DPC. (*D*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from the same cell at rest, during forskolin stimulation alone and after blocker application. Each value represents the mean ± SE of (n) cells obtained from 7 different monolayers.

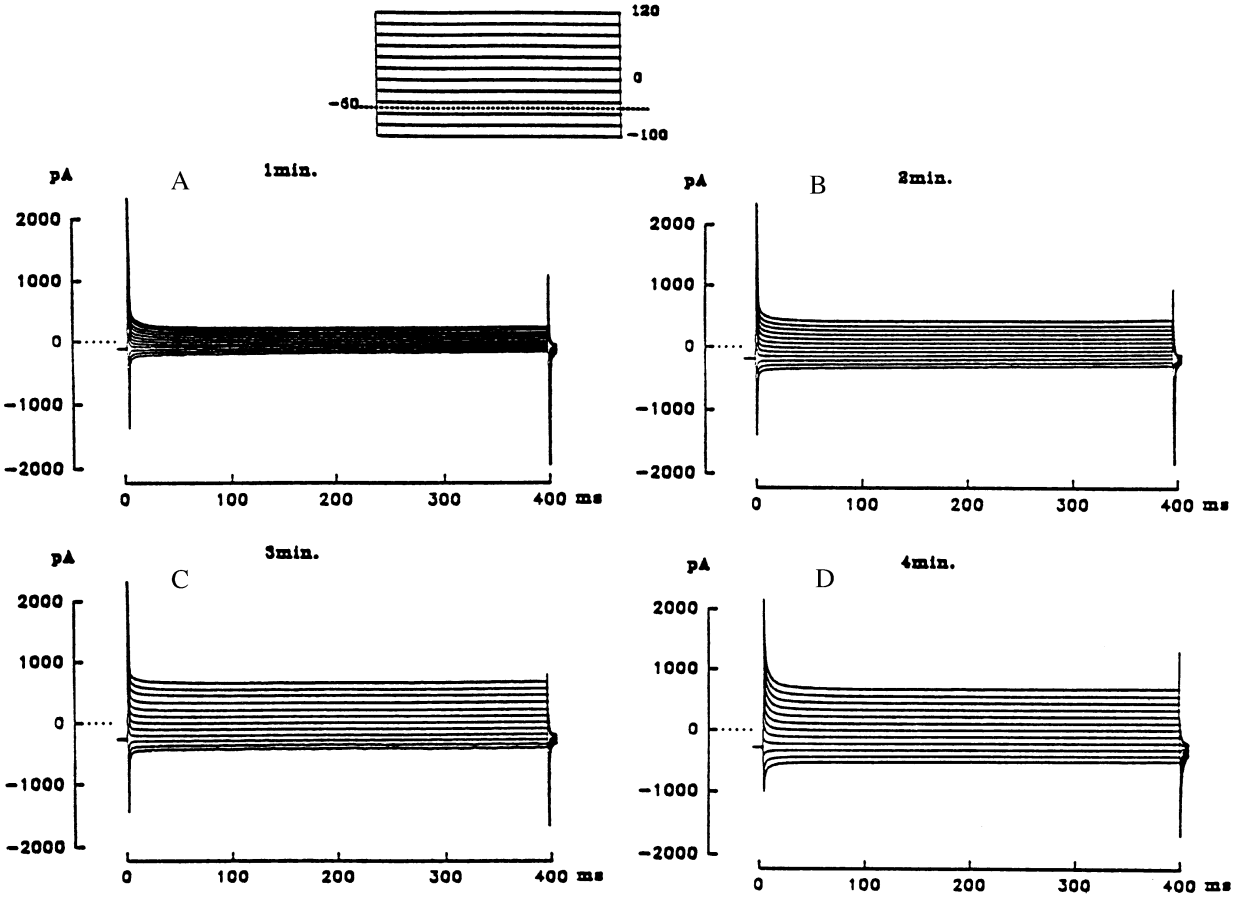

Fig. 5. Characteristics of calcitonin-induced whole cell currents. After obtaining the whole cell clamp configuration, 10^{-7} M calcitonin was perfused and whole cell currents were recorded 1, 2, 4 and 4 min after continuous application of the hormone. The bath contained NaCl solution and the pipette contained NMDGCl solution. The pulse protocol is shown in the top inset.

blocked (by 77 ± 3%, $n = 5$) in the presence of 10^{-3} *M* DIDS (Fig. 10*C* and *E*).

Effects of Forskolin and Calcitonin on Apical and Basolateral Chloride Permeabilities

The Cl^- permeabilities of apical and basolateral membranes were estimated separately by the measurement of intracellular SPQ fluorescence on confluent monolayers. Confluency was checked by the measurement of transepithelial voltage and resistance. V_t averaged −2.94 ± 0.30 mV apical negative and R_t averaged 516 ± 46 $\Omega \cdot cm^2$, $n = 20$. The flows of Cl^- across apical and basolateral membranes were assessed by the addition or removal of Cl^- from either the apical or basolateral solutions.

Apical Membrane

The cell monolayer was first perfused with NaCl solution in both apical and basolateral compartments. After 5 min, the apical Cl^- was replaced by $NaNO_3$ solution and, to block basolateral Cl^- permeability (*see below*), 10^{-4} M NPPB added to the basolateral NaCl solution. Figure 11*A* shows the time course of intracellular SPQ fluorescence. Upon apical Cl^- removal, the SPQ relative fluorescence increased slowly due to Cl^- efflux, then, when NO_3^- was replaced by Cl^-, it fell to approximately the initial level. Addition of forskolin promptly increased the rates of Cl^- efflux and influx previously induced by the Cl^- removal and addition. The initial rate of Cl^- efflux and influx are given in Table 3. Apical application of 10^{-5} M NPPB impaired the forskolin-induced increases of both apical fluxes whereas the addition of apical furosemide remained without significant effect.

The effects of forskolin on apical bromide influx were also tested (Fig. 11*B*): the intracellular Cl^- was first decreased from 29.4 ± 4.1 mM to 4.6 ± 2.1 ($n = 10$) by apical and basolateral NO_3^- substitution. The replacement of apical NO_3 by Br^- caused the SPQ fluorescence to decrease slowly. This decrease reflected the quenching of SPQ due to apical influx of Br^- at a rate of 0.018

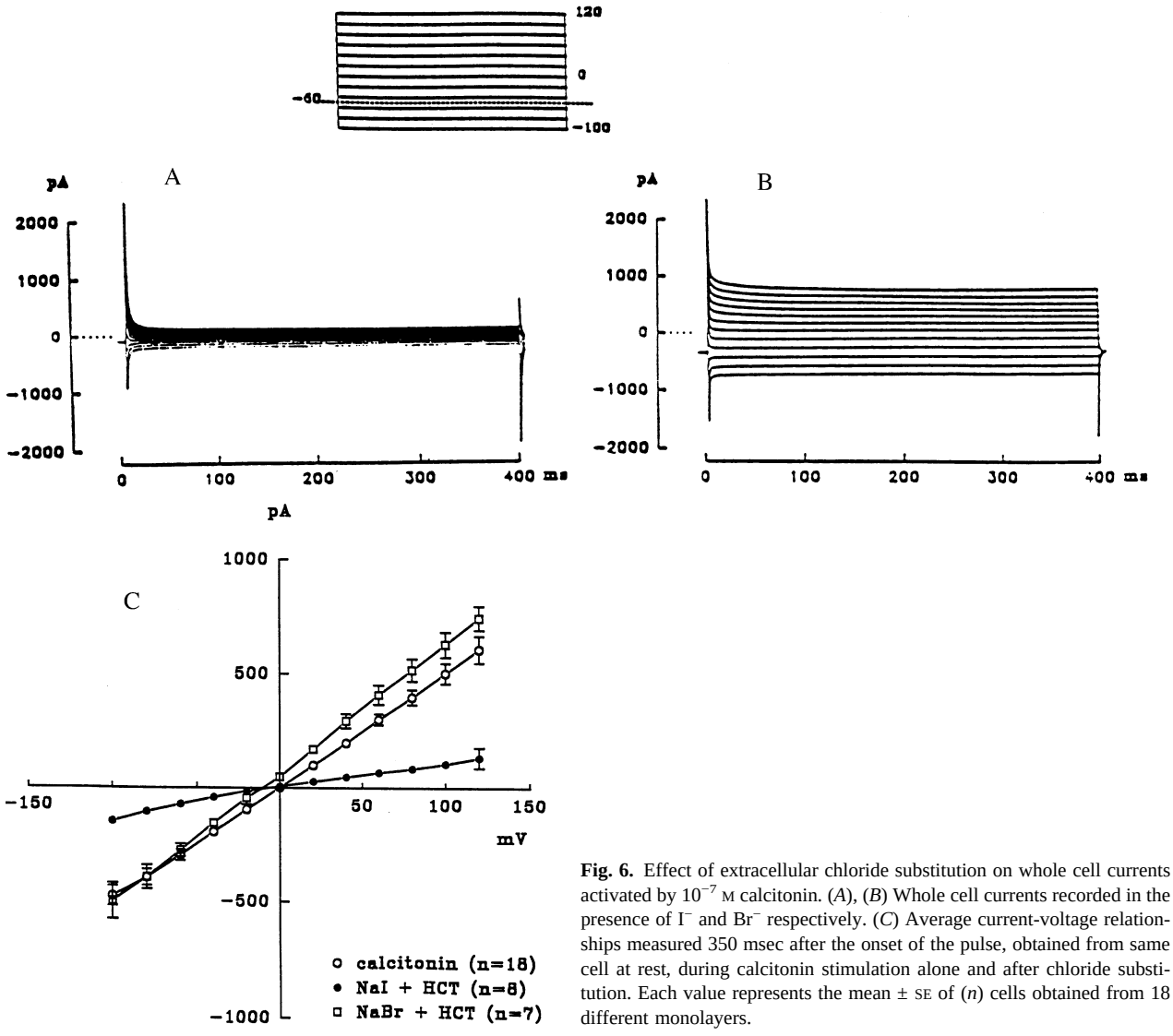

Fig. 6. Effect of extracellular chloride substitution on whole cell currents activated by 10^{-7} M calcitonin. (*A*), (*B*) Whole cell currents recorded in the presence of I^- and Br^- respectively. (*C*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from same cell at rest, during calcitonin stimulation alone and after chloride substitution. Each value represents the mean ± SE of (*n*) cells obtained from 18 different monolayers.

± 0.003 mM Br^- . sec^{-1}. Addition of forskolin during Br^- substitution further enhanced this Br^- influx (0.044 ± 0.002 mM Br^- . sec^{-1}, $P < 0.01$, $n = 5$).

To determine whether calcitonin regulates apical Cl^- efflux, cells were subjected to chloride gradients across the apical membrane in the presence and absence of human calcitonin (10^{-7} M) in the basolateral solution. Throughout the experiments, the basolateral solution contained 10^{-4} M NPPB. Figure 11*C* illustrates the fluorescence changes and Table 3 summarizes the initial rates of Cl^- efflux and influx. Subjection to calcitonin elicited a significant increase in apical Cl^- permeability, Cl^- efflux increasing 1.7-fold and Cl^- influx 1.5-fold. In the series of experiments described in Fig. 12*A*, *B*, and effect of forskolin was tested without Cl^- substitution, the cells being kept in NaCl solution. Forskolin addition induced a significant increase in SPQ relative fluorescence (Fig. 12*A*). In these conditions, the initial rate of Cl^- efflux (0.0050 ± 0.001 mM . sec^{-1}, $n = 3$) was 13 times lower than that measured in the presence of NO_3^- (Table 3). In the presence of 10^{-5} M NPPB in the apical solution, forskolin initiated a decrease in SPQ fluorescence (Fig. 12*B*).

Basolateral Membrane

The basic protocol for studying basolateral Cl^- permeability was identical to that described above for apical permeability studies except that ion substitutions were made in the basolateral solution in the presence of apical NaCl solution containing 10^{-4} M NPPB. In unstimulated monolayers replacement of Cl^- by NO_3^- resulted in a considerable increase in SPQ fluorescence (Fig. 11*D*), indicating that the resting Cl^- permeability was high. The initial rates of basolateral Cl^- efflux are reported in

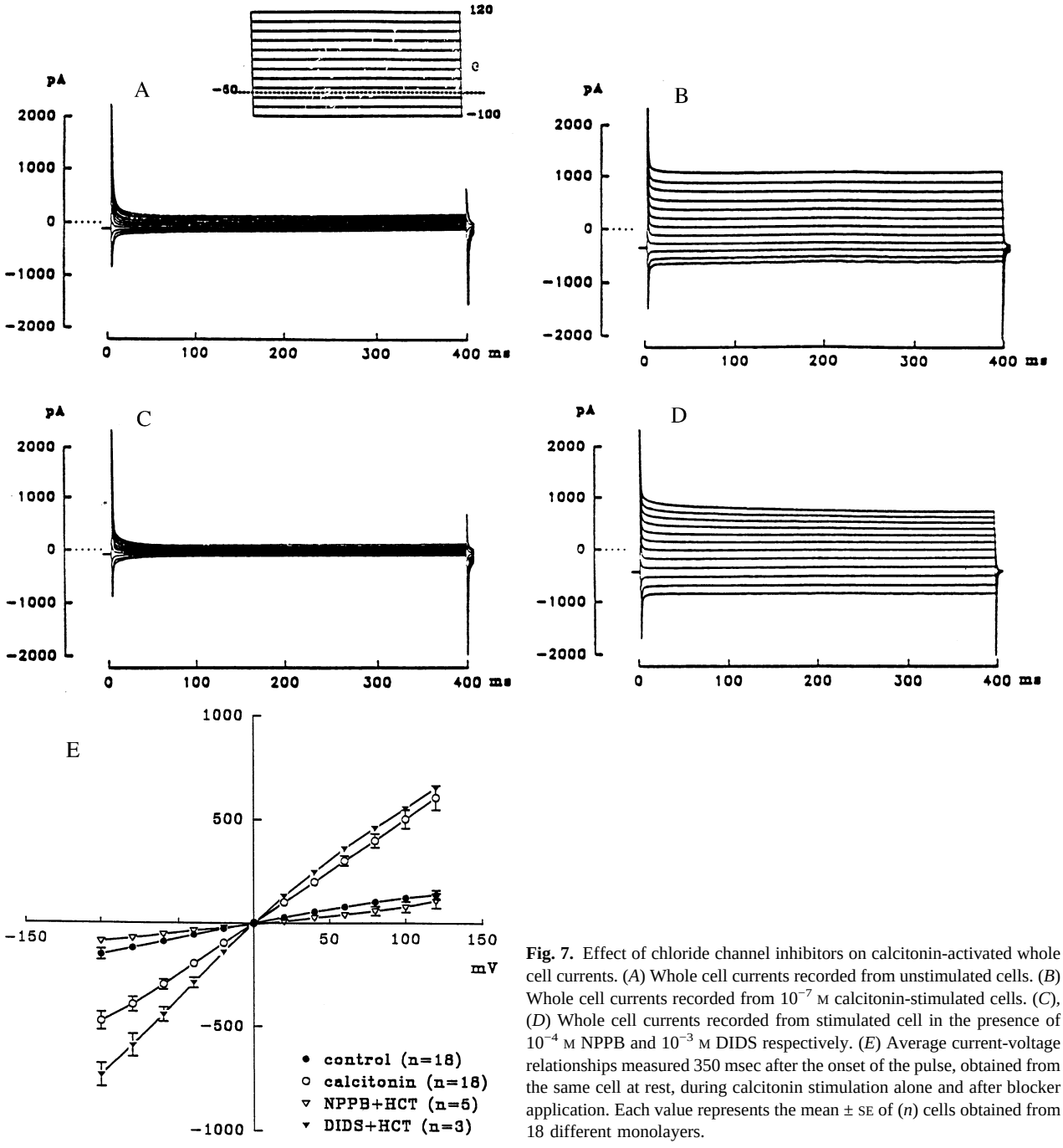

Fig. 7. Effect of chloride channel inhibitors on calcitonin-activated whole cell currents. (*A*) Whole cell currents recorded from unstimulated cells. (*B*) Whole cell currents recorded from 10^{-7} M calcitonin-stimulated cells. (*C*), (*D*) Whole cell currents recorded from stimulated cell in the presence of 10^{-4} M NPPB and 10^{-3} M DIDS respectively. (*E*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from the same cell at rest, during calcitonin stimulation alone and after blocker application. Each value represents the mean ± SE of (*n*) cells obtained from 18 different monolayers.

Table 4. This Cl^- efflux was 3.25 times higher through the basolateral than through the apical membrane. This permeability was not significantly modified by 10^{-5} M NPPB. However increasing the NPPB concentration to 10^{-4} M, inhibited the Cl^- efflux by 66%. As observed in the apical membrane, furosemide did not decrease the basolateral Cl^- efflux (Table 4). Figure 11*D* also shows that addition of forskolin during extracellular Cl^- removal did not induce a further increase of SPQ fluorescence. The calculated effluxes in the presence and absence of forskolin were not significantly different (Table 4).

To estimate the involvement of nonconductive Cl^- pathways in chloride transport across the basolateral membrane, the effects of furosemide and hydrochlorothiazide were investigated. To decrease intracellular Cl^- activity, monolayers were first incubated for 10 min in $NaNO_3$ solution. Basolateral Cl^- influxes were then in-

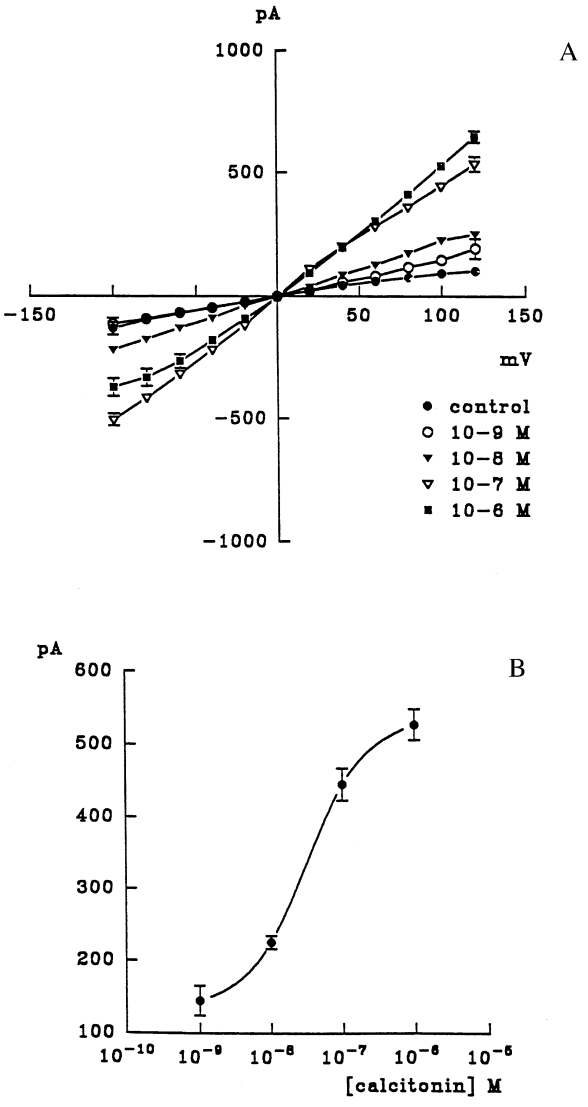

Fig. 8. Dose dependency of the effect of calcitonin on chloride whole cell currents. (*A*) Current-voltage relationships of chloride currents after stimulation by calcitonin (10^{-9} to 10^{-6} M). Currents were measured 4 min after the beginning of the hormone perfusion. (*B*) Dose-response relationship of effect of calcitonin. Each value represents the mean ± SE of 5 experiments performed on different monolayers.

duced by replacing $NaNO_3$ by NaCl. The histogram of Fig. 13 indicates that basolateral application of 10^{-4} M furosemide did not significantly modify the Cl^- influx (control: 0.065 ± 0.017; furo: 0.064 ± 0.009, NS, $n = 3$). A similar result was obtained when using 10^{-3} M hydrochlorothiazide (control: 0.051 ± 0.006; HCTZ: 0.053 ± 0.010, NS, $n = 3$). To confirm the absence of Na^+-dependent chloride transports, Cl^- influx was measured in the absence of basolateral Na^+. Removal of Na^+ did not significantly change the rate of basolateral Cl^- influx (control: 0.061 ± 0.007; NMDGCl: 0.055 ± 0.012, NS, $n = 4$).

Discussion

In the present study, the experiments using the patch clamp technique to measure whole cell currents in cultured DCTb clearly demonstrate that forskolin, cAMP derivatives and calcitonin induce Cl^- currents. Chloride currents were recorded at 33°C with 1 mM EGTA and 5 mM MgATP in the pipette solution. In unstimulated cells, the amplitude of the currents was small and the extracellular perfusion of forskolin and 8 bromo-cAMP activated a linear current. Most of the current recorded in stimulated cells was carried by Cl^- as confirmed by the strongly reduced outward current recorded after the removal of extracellular Cl^-. Although the reversal potential was near to zero in symmetrical Cl^- solutions, the nature of the inward current was unclear. In fact, experiments were generally carried out in the presence of extracellular NaCl but to eliminate the participation of a putative inward Na^+ current, we also performed control experiments either without external Na^+ or with Na^+ solutions containing 10^{-5} M amiloride. In these conditions, the stimulated inward current was never modified, confirming its chloride nature.

The halide selectivity sequence makes it possible to recognize the different types of Cl^- channel. In DCTb cells the sequence for the forskolin-stimulated Cl^- current was $Br^- > Cl^- \gg I^-$. Our data are consistent with a low iodide relative permeability and with an inhibitory effect of I^-, as in other Cl^- channels including CFTR (Cliff, Schumacher & Frizzell, 1992). The sensitivity to various anion channel blockers is also an indication of the nature of the channels. We therefore tested the effects of NPPB, DPC, DIDS. Of three, DPC had the greatest inhibitory effect. In renal tissue as in most epithelial cells, this effect of DPC is drastic irrespective of the nature of the Cl^- conductance. In cultured DCTb, the efficacy of 10^{-4} M NPPB in inhibiting the stimulated Cl^- current was relatively low (70%). However, half inhibitory doses of NPPB vary in different tissues (Sakai et al., 1992). Finally, the forskolin-stimulated Cl^- conductance was quite insensitive to DIDS. Considered together, these results demonstrate that cAMP analogues and forskolin activate a time-independent macroscopic Cl^- current. The linear current-voltage relationship, the anion selectivity sequence and the blocker sensitivity profile, suggest strongly that the macroscopic current recorded in DCTb cells, flows through cAMP-activated CFTR Cl^- channels. We recently demonstrated the existence of a low-conductance, 9pS Cl^- channel in the apical membrane of cultured DCTb (Poncet et al., 1994). This channel displayed a linear current-voltage relationship and moreover, pretreatment of the cells with forskolin or 8Br cAMP increased its expression by about 30%. Thus, the single channel and whole cell currents present very similar features, indicating that low-conductance channels are the predominant channels to be activated by forskolin

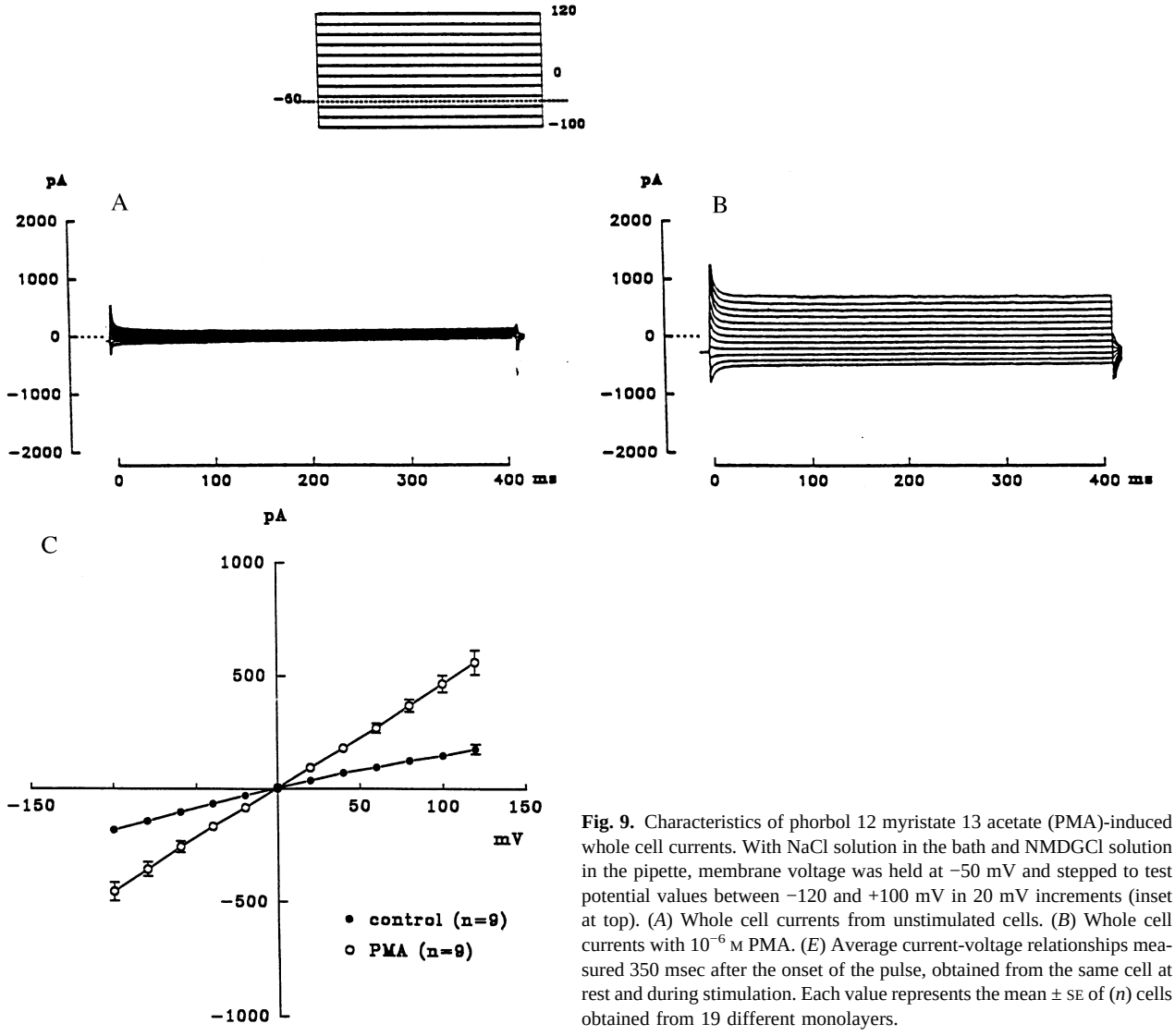

Fig. 9. Characteristics of phorbol 12 myristate 13 acetate (PMA)-induced whole cell currents. With NaCl solution in the bath and NMDGCl solution in the pipette, membrane voltage was held at −50 mV and stepped to test potential values between −120 and +100 mV in 20 mV increments (inset at top). (*A*) Whole cell currents from unstimulated cells. (*B*) Whole cell currents with 10^{-6} M PMA. (*E*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from the same cell at rest and during stimulation. Each value represents the mean ± SE of (*n*) cells obtained from 19 different monolayers.

and cAMP analogues. It is reasonable to assume that activated macroscopic current is the expression of single channels. Hence the 9pS channel provides a whole cell conductance of about 2,000 pS in control conditions and 9,000 pS during forskolin stimulation, which would indicate an opening of about 800 channels per cell.

In view of the fact that calcitonin-sensitive adenylate cyclase activity is confined to the DCTb in the rabbit (Chabardès et al., 1976), the question arises as to whether the hormone induces the same effects as those elicited by cAMP and forskolin. To answer this question, we studied the effect of human calcitonin on while cell currents. In previous studies, we developed the perfusion cell chamber technique to test the effect of the hormone applied on the basolateral side of the culture (Bidet et al., 1990, 1992). In the present work, the cells were superfused with human calcitonin. Exposure to calcitonin was followed within 1–2 min by an increase in whole cell currents in 56% of experiments. In most cells, the calcitonin-stimulated currents, reversed near 0 mV, were time independent, and exhibited an ohmic current-voltage relationship. These properties are very similar to those of the forskolin-activated Cl^- current discussed above. However, the maximal amplitude of the current was lower in the presence of calcitonin than in that of 8 Br cAMP or forskolin. The halide selectivities and blocker effects confirm the identity of the two types of macroscopic current.

The effect of calcitonin was concentration dependent and the IC_{50} value was within the range of the concentration found to stimulate maximally the adenylate cyclase in microdissected DCTb (Chabardès et al., 1976) and cAMP production in cultured DCTb (Mérot et al., 1989). Furthermore, in a previous study we clearly demonstrated that in cultured DCTb cells, calcitonin decreased the cytoplasmic pH by activating the apical Cl^-/

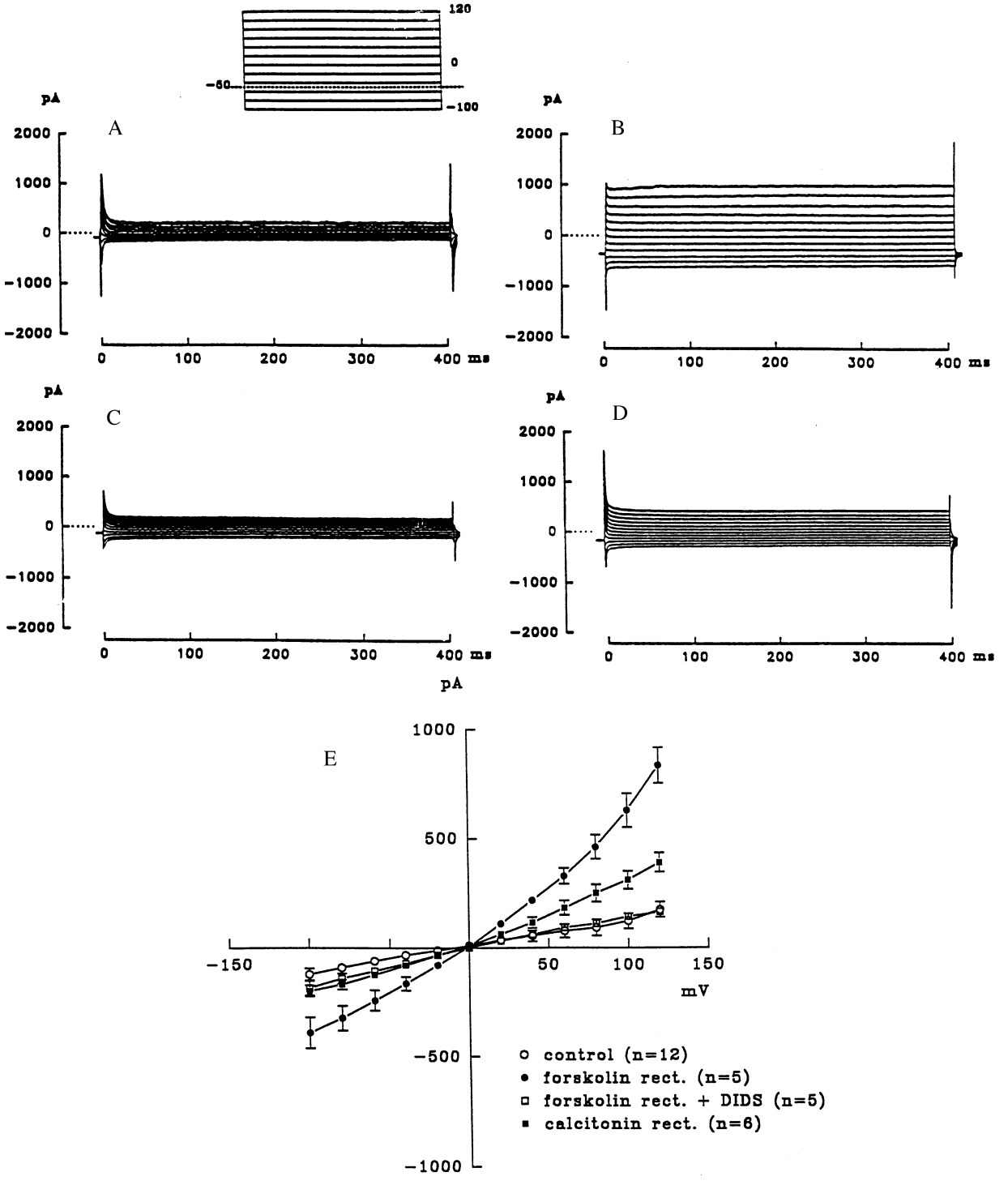

Fig. 10. Outwardly rectifying currents stimulated by forskolin and calcitonin. With NaCl solution in the bath and NMDGCl solution in the pipette, membrane voltage was held at −50 mV and stepped to test potential values between −120 and +100 mV in 20 mV increments (inset at top). (*A*) Whole cell currents for unstimulated cell. (*B*) Whole cell currents for 10^{-5} M forskolin stimulated cells. (*C*) Whole cell currents for forskolin-stimulated cells in the presence of 10^{-3} M DIDS. (*D*) Whole cell currents for 10^{-7} M calcitonin-stimulated cells. (*E*) Average current-voltage relationships measured 350 msec after the onset of the pulse, obtained from the same cell at rest and during stimulation. Each value represents the mean ± SE of (*n*) cells obtained from 12 different monolayers.

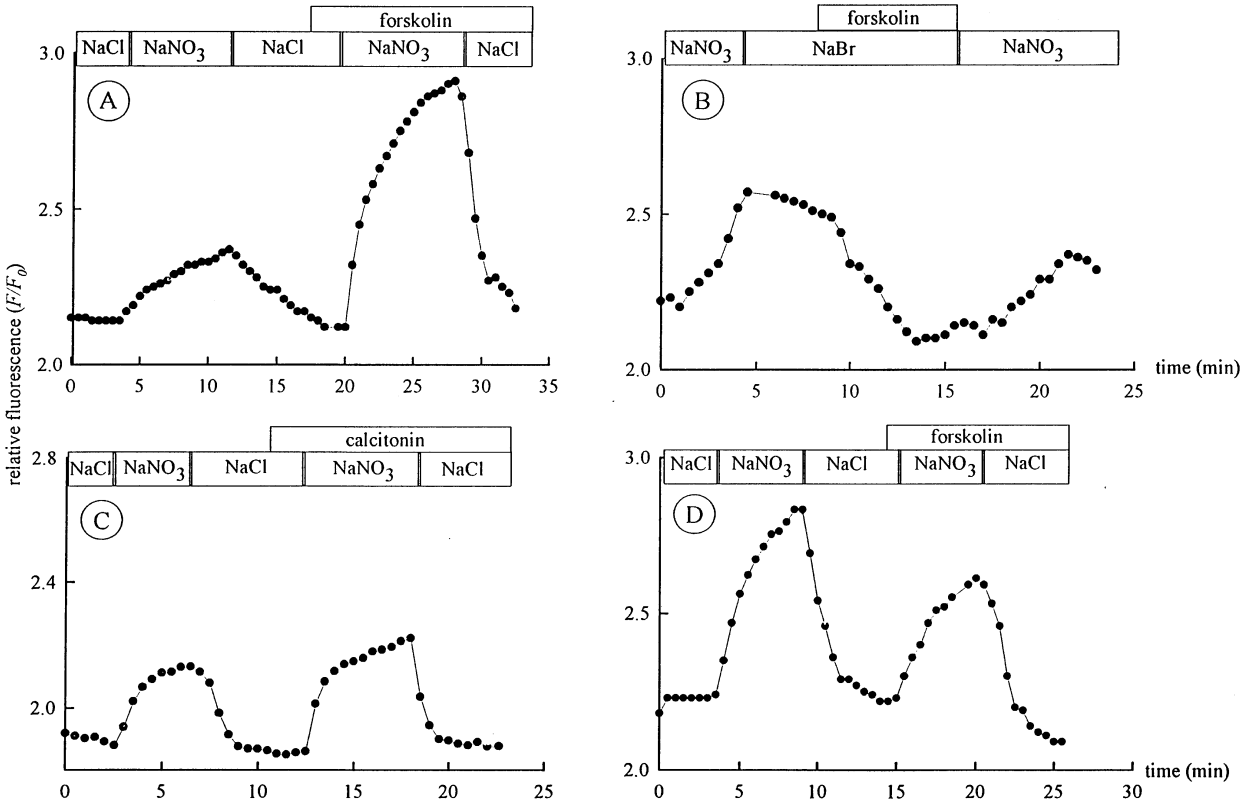

Fig. 11. SPQ fluorescent experiments. (*A*) Effects of forskolin on Cl^- efflux and influx through the apical membrane. The sequence of buffer substitutions in the apical compartment is indicated in the top inset. 10^{-5} M forskolin was added to both compartments 2 min before the second NaCl substitution by $NaNO_3$. The basolateral compartment was continuously perfused with the NaCl solution containing 10^{-4} M NPPB. (*B*) Effects of forskolin or Br^- influx through the apical membrane. 140 mM NaBr was perfused in the apical compartment and 10^{-5} M forskolin was added in both compartments as indicated. The basolateral compartment was continuously perfused with the $NaNO_3$ solution containing 10^{-4} M NPPB. (*C*) Effects of calcitonin of Cl^- efflux and influx through the apical membrane. The sequence of buffer substitutions in the apical compartment is indicated in the top inset. 10^{-7} M calcitonin was added to the basolateral compartment 2 min before the second NaCl substitution. The basolateral compartment was continuously perfused with the NaCl solution containing 10^{-4} M NPPB. (*D*) Effects of forskolin on Cl^- efflux and influx through the basolateral membrane. The sequence of buffer substitutions in the basolateral compartment is indicated in the top inset. 10^{-5} M forskolin was added in both compartments 2 min before the second NaCl substitution. The apical compartment was continuously perfused with the NaCl solution containing 10^{-4} M NPPB. The results are expressed in relative fluorescence F/F_0 where F is the fluorescence in function of time and F_0 the value of the fluorescence obtained after total SPQ fluorescence quenching by KSCN. Each point corresponds to the average fluorescence of 20 to 30 cells.

HCO_3^- exchanger with an IC_{50} of 4 10^{-8} M (Bidet et al., 1992). This action was also mimicked by cAMP-permeant derivatives and forskolin. Taken all together, these observations suggest that calcitonin action in increasing Cl^- currents and activating the Cl^-/HCO_3^- exchanger, may be mediated by cAMP via an adenylate cyclase stimulation. However, in the present study, preliminary experiments using PMA, indicate that PKC could be involved in the regulation of whole cell Cl^- currents. At present, we have not pursued our investigation into the exact nature of this macroscopic Cl^- conductance stimulated by PMA, although the Cl^- current appeared to resemble that induced by forskolin, cAMP derivatives and calcitonin. One interesting hypothesis would be that calcitonin controls the small-conductance Cl^- channel by both the PKA and the PKC pathways. Of course, this is only a working hypothesis but it is noteworthy that molecular cloning experiments have clearly established that the calcitonin receptor is coupled to adenylate cyclase and phospholipase C (Chabre et al., 1992; Force et al., 1992). Moreover, a number of studies have implicated activation of the same Cl^- conductance by PKC and PKA (Suzuki et al., 1991; Tabcharani et al., 1991; Dechecchi et al., 1993; Sahi et al., 1994).

In some cases, forskolin and calcitonin induced an outwardly rectifying Cl^- conductance which was clearly blocked by DIDS. Because this conductance was only rarely recorded, no extensive experiments could be performed. It is however, very unlikely that the stimulated current was activated by an increase in Ca^{2+} concentration since first, an EGTA concentration as high as 10 mM in the pipette solution did not impair the forskolin re-

Table 3. Forskolin and calcitonin stimulated fluxes through the apical membrane

	Forskolin ($n = 16$)		Forsk. + NPPB ($n = 5$)	
	Control	Experiment	Control	Experiment
Efflux mmol · Cl^- · sec^{-1}	0.016 ± 0.006	0.067 ± 0.020	0.013 ± 0.006	0.007 ± 0.004
	$P < 0.01$		*NS*	
Influx mmol · Cl^- · sec^{-1}	0.030 ± 0.006	0.061 ± 0.010	0.016 ± 0.005	0.013 ± 0.003
			NS	
	$P < 0.01$			
	Forsk. + Furo ($n = 6$)		Calcitonin ($n = 7$)	
	Control	Experiment	Control	Experiment
Efflux mmol · Cl^- · sec^{-1}	0.016 ± 0.003	0.047 ± 0.009	0.038 ± 0.003	0.065 ± 0.01
	$P < 0.05$		$P < 0.05$	
Influx mmol · Cl^- · sec^{-1}	0.011 ± 0.002	0.023 ± 0.003	0.052 ± 0.003	0.078 ± 0.009
			$P < 0.05$	
	$P < 0.01$			

Initial rates of Cl^- efflux and influx were determined after removal and addition of Cl^- in the solution bathing the apical membrane. The solution bathing the basolateral membrane contained 140 mM and 10^{-4} M NPPB. Values are means of (n) experiments performed on different monolayers. Student's paired t test was used for statistical analysis.

sponse and second, in a previous study we demonstrated that forskolin does not modify the intracellular calcium concentration (Poujeol, Bidet & Tauc, 1995). Increase in cell volume can also be discarded as a stimulating factor because the bathing solution was made hyperosmotic by addition of 60 mM mannitol to prevent swelling-activated Cl^- currents (Worrel et al., 1989). A recent study has described ORCC (Outwardly Rectifying Chloride Channel) currents in cultured human airway epithelial cells (Schwiebert, Lopes & Guggino, 1994). It is interesting that these authors demonstrated that both CFTR Cl^- channels and ORCC contribute to cAMP activated whole cell Cl^- currents. Up to now, we do not know whether the two types of Cl^- channel are present together in the same cell. In spite of the fragmentary nature of the information we obtained concerning ORCC, our results corroborate the observations of Schwiebert et al. (1994). Our previous patch clamp study of cultured DCTb cells demonstrated the presence of a rarely expressed larger conductance Cl^- channel with an outwardly rectifying *I-V* relationship. The identity of this channel with ORCC remains to be proved.

Whole cell clamp analysis is a useful technique for studying ion conductance but does not permit a precise location of the recorded current on the membrane. By using patch clamp analysis we have identified the small conductance Cl^- channel in the apical membrane of cultured DCTb cells. In this work, the cultures grew on collagen-coated Petri dishes and the question arose as to whether this support allowed the cells to polarize normally. Moreover, the basolateral membrane was not readily accessible in these cultures and no direct data could be obtained on putative basolateral ion channels. To investigate the membrane location of the Cl^- conductances, we therefore developed the technique of SPQ quantitative fluorescence measurement in DCTb monolayers grown on permeable collagen-coated filters (Bidet et al., 1990). Intracellular Cl^- activity had been measured by SPQ in many tissues (Chao, Widdicombe & Verkman, 1990; Halm, Kirk & Sathiakumar, 1993; Dho & Foskett, 1993; Lu, Markakis & Guggino, 1993). This reliable technique provides information about both conductive and nonconductive pathways. Our results show that forskolin only increases the Cl^- permeability in the apical membrane. This forskolin effect was completely abolished in the presence of NPPB but not modified by furosemide, demonstrating the conductive nature of the Cl^- fluxes. The factor of increase of the forskolin-sensitive Cl^- efflux was very close to that of the forskolin-sensitive macroscopic Cl^- currents (SPQ: 4.2; Whole cell: 4.4). Clearly, the two experimental approaches reveal the same biological phenomenon which is control of the small Cl^- channels by PKA only in the apical membrane. The two methods also demonstrated that in addition to forskolin, calcitonin increases the apical Cl^- conductance.

Ionic substitution at the basolateral side of the monolayer showed that a Cl^- pathway exists at this site. The basolateral permeability was found to be about 3 times higher than the apical Cl^- permeability and NPPB sensitivity ten times lower. Such a differential Cl^- permeability has already been postulated in the thin ascending limb of Henle's loop (Yoshitomi, Kondo & Imai, 1988). The Cl^- influx in the presence of Cl^- gradients was not

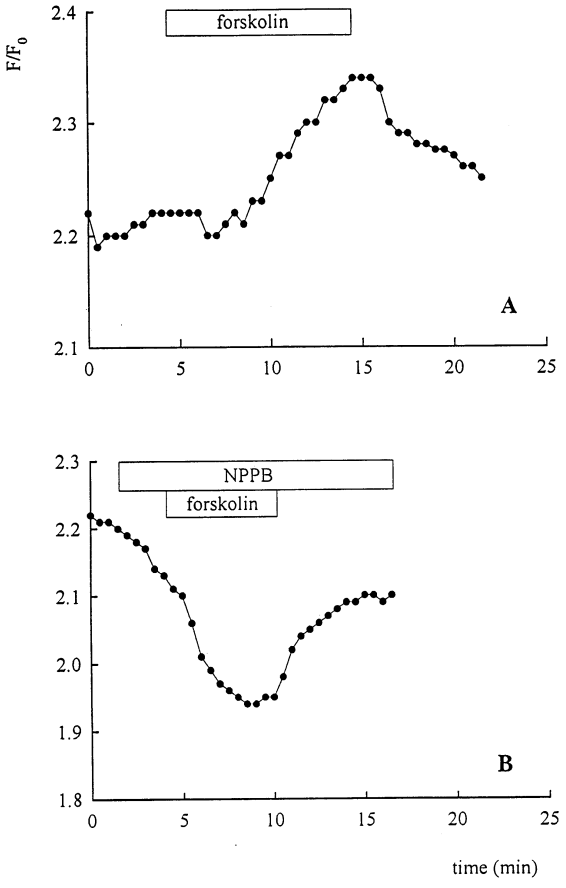

Fig. 12. Effects of forskolin and NPPB on intracellular Cl^- activity in SPQ-loaded DCTb cells. (*A*) SPQ-loaded cells were incubated in NaCl solution in both compartments. 10^{-5} M forskolin was added during the period indicated. (*B*) Similar experiment but with 10^{-4} M NPPB in the apical compartment. The results are expressed in relative fluorescence F/F_0 where F is the fluorescence in function of time and F_0 the value of the fluorescence obtained after total SPQ fluorescence quenching by KSCN. Each point corresponds to the average fluorescence of 20 to 30 cells.

sensitive to furosemide, hydrochlorothiazide and sodium removal indicating that Na-K-2Cl and Na-Cl cotransports do not occur in the basolateral membrane. Finally, the basolateral Cl^- permeability was not sensitive to forskolin.

A number of studies have demonstrated that Cl^- channels in both secreting and reabsorbing epithelia are stimulated by cAMP. In the former, it has now been clearly established that the Cl^- conductance associated with the small linear Cl^- channel sensitive to cAMP, correlates with the expression of the CFTR. This channel is located unequivocally in the apical membrane (Frizzell & Morris, 1994). The cAMP-dependent regulation could be direct via phosphorylation of membrane-resident CFTR and indirect via an increase in CFTR insertion into the apical membrane (Frizzel & Morris, 1994). In the kidney, several Cl^- channels have already been described. Each channel is discussed in detail in a very complete review by Schwiebert et al. (1994). Up to now, a small conductance cAMP-sensitive Cl^- channel sharing properties with the CFTR Cl^- channels has been found in the apical membrane of monolayers obtained from rabbit DCTb (Poncet et al., 1994), CCD (Ling & Kokko, 1992) and IMCD (Vandorpe et al., 1993) as well as in the A6 cell line (Marunaka & Tohda, 1993). Our data also suggest that CFTR exists in the apical membrane of rabbit DCTb in primary culture. Using RT-PCR techniques, we have recently shown that rabbit CFTR is strongly expressed in cultured DCTbs (Tauc et al., 1995). However, further studies using immunofluorescence techniques remain to be carried out to determine the exact membrane location of the protein.

The existence of Cl^- channels in the basolateral membrane of the nephron is beginning to be well documented. Gesek & Friedman (1993) postulated an NPPB-sensitive Cl^- conductance in the basolateral membrane of an immortalized cell line derived from mouse DCT and suggested that calcitonin increased the anion permeability. In the present study, we found that the calcitonin effect of increasing Cl^- conductance via the cAMP cascade was only present in the apical membrane and that cAMP did not control the Cl^- permeability of the basolateral membrane. Furthermore, our data do not indicate an action of calcitonin on the basolateral Cl^- permeability. Other workers have concluded that specific Cl^- channels may be expressed in both apical and basolateral membranes (Schwiebert et al., 1994). However, concerning the regulation by polypeptide hormones via the cAMP/PKA pathway, it is tempting to consider that the activated Cl^- conductive pathway is mainly located in the apical membrane. This is true for calcitonin in DCTb, it is also true for PTH in PCT (Suzuki et al., 1993), vasopressin in CCD (Nagy, Naray-Fejes-Toth & Fejes-Toth, 1994), IMCD (Kizer et al., 1993) and A6 cell lines (Marunaka & Tohda, 1993). Except in PCT, the channels involved resemble CFTR channels but additional experiments will be necessary to confirm the correlation between apical CFTR and the cAMP-activated Cl^- conductance response.

In cultured DCTb, the physiological role of an apical Cl^- conductance in series with a basolateral Cl^- conductance remains unclear. *In vivo,* however, the apical Cl^- concentration in the distal fluid is low and calcitonin might induce Cl^- secretion. Since this hormone could also increase the activity of the apical Cl^-/HCO_3^- exchanger (Bidet et al., 1992), the consequence would be an increase of HCO_3^- secretion with a recycling of Cl^- through the apical membrane.

In conclusion, the present study confirms the presence of a Cl^- conductance sensitive to cAMP derivatives and forskolin in cultured DCTb cells. This conductance is located exclusively in the apical membrane and is

Table 4. Cl^- efflux through the basolateral membrane

	NPPB (n = 3)			Furosemide (n = 4)		Forskolin (n = 5)	
	Control	10^{-5} M	10^{-4} M	Control	Experiment	Control	Experiment
Efflux mmol · Cl^- · sec^{-1}	0.052 ± 0.002 *NS*	0.046 ± 0.003 $p < 0.01$	0.018 ± 0.004	0.065 ± 0.003 *NS*	0.062 ± 0.003	0.070 ± 0.01 *NS*	0.053 ± 0.01

Initial rate of Cl^- efflux was determined after removal of Cl^- in the solution bathing the basolateral membrane. The solution bathing the apical membrane contained 140 mM NaCl and 10^{-4} M NPPB. Values are means of (n) experiments performed on different monolayers. Student's paired t test was used for statistical analysis.

increased by calcitonin probably by the cAMP/PKA pathway, although PKC intervention is also probable. The main channel involved may be the small conductance Cl^- channel corresponding to CFTR-like channels. However, a few experiments indicated an activation by forskolin and calcitonin of a slightly outwardly rectifying Cl^- conductance, resembling ORCC. In basal conditions, the basolateral membrane is more conductive to Cl^- than is the apical membrane. Basolateral Cl^- permeability is not controlled by the cAMP pathway and its exact nature is not known. Recent experiments undertaken in our laboratory demonstrate the existence of Ca^{2++}-dependent and volume-activated Cl^- channels (Tauc, Bidet & Poujeol, 1995). An attractive working hypothesis would be that these channels account for the basolateral Cl^- conductance.

Recent studies on airway epithelia suggest some link between the function of CFTR and Na^+ channels (Chinet et al., 1994; Grubb, Vick & Boucher, 1994). In previous studies, we identified amiloride-sensitive Na^+ channels in the apical membrane of cultured DCTb cells (Mérot et al., 1989, 1991). Moreover, in these cells, calcitonin may decrease the apical Na^+ conductance (Mérot et al., 1989). Until future studies characterize further the physiological role of CFTR in DCTb, we suggest that calcitonin controls the function of CFTR Cl^- channels and that CFTR acts by regulating, perhaps in an inhibitory fashion, Na^+ channel activity.

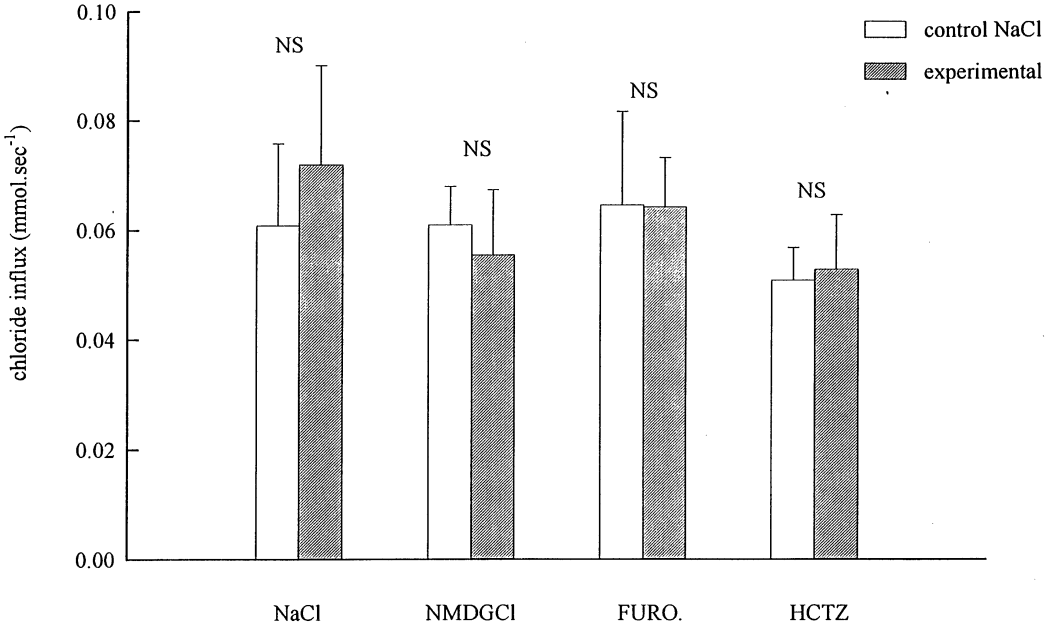

Fig. 13. Effects of furosemide, hydrochlorothiazide and the absence of sodium on the Cl^- influx through the basolateral membrane. SPQ-loaded DCTb cells were perfused in both compartments with $NaNO_3$ solution for 10 min to reduce the intracellular Cl^- concentration. After which, 140 mM NMDGCl (n = 4) or 10^{-4} M furosemide (n = 3) or 10^{-3} M hydrochlorothiazide (n = 3) in 140 mM NaCl were perfused in the apical compartment. Open bars represent the values of the Cl^- influx in control conditions (n = 4) and hatched bars the experimental conditions. Values (mmol . sec^{-1}) are means ± SE.

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