Modulation of the hyperpolarization-activated Cl^- current in human intestinal T_{84} epithelial cells by phosphorylation

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1. Hyperpolarization-activated Cl^- currents ($I_{Cl,hyp}$) were investigated in the T_{84} human adenocarcinoma cell line, using the patch-clamp whole-cell configuration.

2. During whole-cell recording with high-chloride and ATP-containing internal solutions, hyperpolarizing jumps from a holding potential of 0 mV elicited slow inward current relaxations, carried by Cl^- and detected at membrane potentials more negative than −40 mV. Analysis of the relative permeabilities to monovalent anions gave the following sequence: $Cl^- > Br^- > I^-$ > glutamate.

3. $I_{Cl,hyp}$ was partially inhibited by 1 mM diphenylamine-2-carboxylic acid or 0·1 mM 5-nitro-2-(3-phenylpropylamino)-benzoate, and was completely blocked by Cd^{2+} (>300 μM). It was insensitive to 1 mM external 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid or 1 mM Ba^{2+}.

4. $I_{Cl,hyp}$ was inhibited by external application of 500 μM cptcAMP (8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate) or 500 nM of the protein kinase C activator, phorbol 12-myristate, 13-acetate .

5. (i) Omission of ATP from the pipette solution, (ii) ATP replacement by the non-hydrolysable ATP analogue 5′-adenylylimidodiphosphate, and (iii) inhibition of protein kinase C by staurosporine or calphostin C accelerated the activation kinetics of the current and increased its amplitude, but did not alter its pharmacological properties.

6. We conclude that hyperpolarization-activated Cl^- channels similar to those of ClC-2 channels (mammalian homologue of *Torpedo* chloride channel ClC-0) are present in T_{84} cells, and that their gating properties are modulated by phosphorylation.

Chloride channels in the apical membrane play a central role in the secretion of electrolytes and fluid across the epithelia. The currently accepted model for Cl^- transport across secretory epithelia, such as those of the airways and intestine, consists of concerted mechanisms involving the uptake of Cl^- via basolateral membrane cotransporters and Cl^- efflux through the Cl^- channels located at the apical membrane (Quinton, 1990; Anderson, Sheppard, Berger & Welsh, 1992). Dysfunction in the regulation of these channels results in abnormal fluid secretion and underlies such diseases as cystic fibrosis or secretory diarrhoea (Field & Semrad, 1993).

The T_{84} intestinal cell line has been extensively used to investigate the mechanisms of chloride secretion. Both cAMP and Ca^{2+} stimulate this secretion in T_{84} cells, but appear to do so by somewhat different mechanisms (Barrett, 1993). It is now established that the cystic fibrosis transmembrane regulator (CFTR) constitutes the apical chloride channel involved in secretion in response to hormones which raise the levels of intracellular cAMP (Anderson *et al.* 1992; Fuller & Benos, 1992). The pathways involved in the Ca^{2+}-dependent Cl^- secretion have been less well defined. Ca^{2+}-activated chloride channels have been described in isolated T_{84} cells (Cliff & Frizzell, 1990) but are not present in confluent polarized cells (Anderson & Welsh, 1991). Hormones that raise $[Ca^{2+}]$ are believed to stimulate basolateral K^+ conductance thereby hyperpolarizing the cell membrane potential and increasing the driving force for chloride exit through unidentified apical chloride channels that are constitutively opened (Anderson *et al.* 1992; Barrett, 1993).

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Chloride channels activated by hyperpolarization have been described in several non-epithelial and epithelial cell types. They have been well characterized in the electric organ of *Torpedo* (Miller & Richard, 1990), and *Aplysia* neurons (Chesnoy-Marchais, 1983). Subsequently, the presence of voltage-activated chloride conductance was reported in vertebrate neurons (Madison, Malenka & Nicoll, 1986), oocytes (Taglietti, Tanzi, Romero & Simoncini, 1984; Parker & Miledi, 1988; Kowdley, Ackerman, John, Jones & Moorman, 1994), ascidian embryos (Block & Moody, 1990), lymphocytes (Pahapill & Schlichter, 1992), and osteoblasts (Chesnoy-Marchais & Fritsch, 1994). Single channel recordings from collecting duct basolateral membrane (Sansom & Carosi, 1990) have also revealed chloride channels slowly activated by hyperpolarization. A voltage-activated chloride conductance was recently described in the granular duct cells of mouse mandibular gland (Dinudom, Young & Cook, 1993; Komwatana, Dinudom, Young & Cook, 1994). Some voltage-activated chloride channels have now been cloned, including the *Torpedo* chloride channel named ClC-0 (Jentsch, Steinmeyer & Schwarz, 1990) and one of its mammalian homologues, ClC-2 (Thiemann, Gründer, Pusch & Jentsch, 1992). The ClC-2 protein has been identified as a chloride channel slowly activated by hyperpolarization when expressed in *Xenopus* oocytes. Northern blot analysis has shown that ClC-2 is expressed in a wide variety of tissues and cell lines, including the T_{84} cell line. The presence of ClC-2 transcripts in T_{84} cells suggested the existence of hyperpolarization-activated Cl^- currents in these cells (Thiemann *et al.* 1992).

In the work reported here, we used the whole-cell patch-clamp technique to demonstrate the presence of a hyperpolarization-activated chloride current ($I_{Cl,hyp}$) in human intestinal T_{84} cells, and to study its pharmacological properties. Our results show that the gating properties of this current can be modulated by ATP hydrolysis and protein kinase C.

METHODS

Cell culture

The T_{84} cell line is a human colonic carcinoma cell line that was obtained from a lung metastase. The cells used in this work were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA) They were grown in Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) containing 10% fetal calf serum, 2 mM glutamine, 100 i.u. penicillin and 100 μg ml^{-1} streptomycin at 37 °C in an atmosphere of 5% CO_2–95% air. Cells were regularly subcultured by enzymatic treatment with a solution of 0·25% trypsin, 1 mg ml^{-1} EDTA in a Ca^{2+}- and Mg^{2+}-free phosphate buffer solution. Cells from passages 52–107 were used 1–3 days after plating on 35 mm Petri dishes (Costar). Culture dishes were slowly perfused with the external solution. A four-barrel fast perfusion system made of glass and Teflon tubing, was used for fast application of the external agents to be tested. The recorded cell was continuously perfused with one of these barrels containing control or test solutions. Experiments were performed on isolated cells at room temperature (21–26 °C).

Solutions

The external standard solution contained (mM): 140 *N*-methyl-D-glucamine chloride (NMGCl), 10 Hepes (pH 7·3 adjusted with NMG), 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 sucrose. Table 1 gives the composition of the internal solutions used.

Whole-cell current recording

Patch-clamp micropipettes were made of hard glass (Kimax 51, Kimble, Toledo, OH, USA). The shank of each pipette was coated with Sylgard and the tip was fire polished. Pipette tip resistance, measured in the external standard solution, was between 4 and 6 MΩ when the pipette was filled with solution 1 (Table 1). The cells were voltage clamped by an Axopatch 200A amplifier (Axon Instruments Inc.) controlled by a 386 computer via a CED1401 interface (Cambridge Electronic Design Ltd, Cambridge, UK), using CED voltage-clamp software. The current monitor output of the amplifier was filtered at 0·1 kHz before being sampled on line at 0·2 kHz. The bath was connected to the ground via an agar bridge; junction potentials between the pipette and bath solutions were measured and corrections made.

Currents were activated by applying hyperpolarizing voltage jumps, from a holding potential of 0 mV. The usual stimulation protocol consisted of regular jumps of 10 or 20 s to −120 mV, separated by 50 s periods at 0 mV. The current–voltage relationship was determined by raising the voltage in 20 mV increments in negative or positive voltage ranges. Leak currents were not subtracted. Membrane capacitance and series resistance were routinely measured using the analog circuitry of the amplifier, and compensated for, before recording started. Cell capacitance was 13–30 pF, and series resistance was 10–15 MΩ when using the high-chloride internal pipette solution (solution 1, Table 1).

When appropriate, data are expressed as means ± S.D.

Pharmacological agents

Diphenylamine-2-carboxylic acid (DPC) and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) were purchased from Fluka (Buchs, Switzerland). 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB) was purchased from Research Biochemicals International. Stock solutions of DPC and NPPB were prepared in dimethyl sulphoxide (DMSO final concentration in the test solution, 0·1%), whereas DIDS was dissolved directly in the external solution. 5′-Adenylylimidodiphosphate (AMPPNP), 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (cptcAMP), forskolin, and staurosporine were purchased from Sigma. A stock solution of forskolin was prepared in ethanol (final concentration of ethanol in the test solution, 0·1%) and kept in the dark at −20 °C. Phorbol 12-myristate, 13-acetate (PMA) and 4α-phorbol 12-myristate, 13-acetate (4αPMA) were purchased from RBI). Stock solutions of PMA and 4αPMA (10 mM) were prepared in DMSO (final concentration of DMSO in the test solution, 0·2%). Calphostin C was obtained from Calbiochem. Stock solutions of staurosporine and calphostin C were prepared in DMSO (final concentration in the test solution, 0·1%). The cAMP- dependent protein kinase inhibitor cAMP DPKI 5–24 amide was obtained from Peninsula Laboratories (Belmont, CA, USA) and dissolved in the internal solution.

Table 1. Composition (in mM) of the internal solutions

Internal solution number	CsCl	Cs^{2+} Glut	NMGCl	NMG Glut	EGTA	$CaCl_2$
1 (145 Cl^-)	30	—	113	—	0·5	0
2 (137 Cl^-)	30	—	101	—	10	1
3 (20 Cl^-)	18	12	—	108	0·5	0
4 (30 Cl^-)	28	—	—	115	0·5	0

NMG, *N*-methyl-D-glucamine; Glut, glutamate. All the internal solutions contained 10 mM Hepes, buffered at pH 7·3 with NMG and 1 mM $MgCl_2$. Unless otherwise specified, they were supplemented with 3 mM MgATP (or in some experiments 3 mM Na_2ATP). The osmolarity of the external solution was 320 mosmol l^{-1}. The final osmolarity of all internal solutions was kept at 285 mosmol l^{-1} (35 mosmol l^{-1} less than that of external solution) to avoid cell swelling, as previously described (Worrell, Butt, Cliff & Frizzell, 1989). Osmolarity was measured using a vapour pressure osmometer (Wescor 5500, Bioblock Scientific, France).

RESULTS

When T_{84} cells were held in the whole-cell configuration with the pipette solution containing high chloride and MgATP or Na_2ATP (solution 1, Table 1), pulses of hyperpolarizing voltage from a holding potential of 0 mV elicited slow inward current relaxations. After equilibration of the intracellular compartment with this solution, the amplitude of the currents recorded in response to regular voltage steps from 0 to −120 mV remained stable for tens of minutes. Successive current traces were recorded (Fig. 1*B*) using the voltage protocol described in the legend to Fig. 1*A*. For voltages more negative than −40 mV, the currents activated slowly and needed 20–30 s of hyperpolarization to reach full activation. Outward currents were negligible in the positive voltage range. Under these experimental conditions, activation of the currents could not be satisfactorily resolved by exponential fits, and current amplitude was therefore estimated by measuring the difference in the amplitudes recorded between 30 ms and 10 s. The inward rectification of the current–voltage relationship shown in Fig. 1*C* reflects the voltage dependence of the underlying activated conductance. Inward current relaxation was detected in 95% of the cells studied, but current amplitude varied considerably, even when it was recorded on cells taken from the same batch and studied on the same day. This variability could not simply be accounted for by variations in cell size, since the current values recorded at −120 mV, normalized and expressed per 20 pF

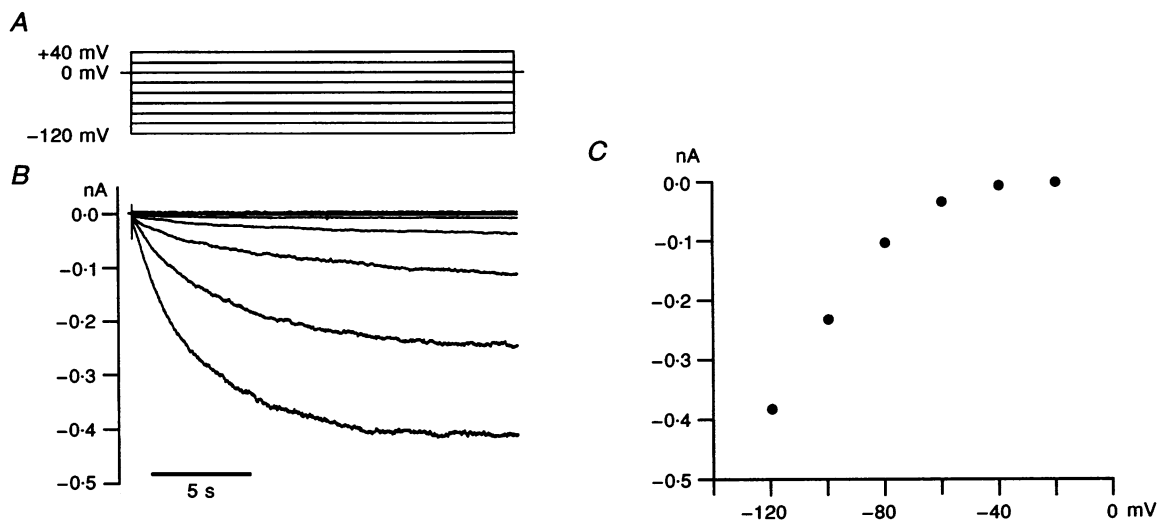

Figure 1. *I–V* curve of the current activated by hyperpolarization

A, voltage jump protocol used to establish the *I–V* curve. Potentials lasting 20 s duration were delivered stepwise in 20 mV increments every 50 s from a holding potential of 0 to −120 mV and to +40 mV. *B*, current traces recorded during the series of voltage jumps in a cell dialysed with high-chloride and ATP-containing internal solution (solution 1, Table 1). *C*, corresponding *I–V* curve of the current relaxation measured as the difference in current amplitudes measured between 10 s and 30 ms.

cell capacitance, ranged from 25 to 900 pA. The histogram of current amplitudes in Fig. 2 shows that they were lower than 200 pA in 66% of the cells studied. Note that the morphological aspect differed from cell to cell. In the course of the experiments, we observed that the largest current amplitudes (>200 pA) were usually recorded in cells that presented a granular appearance under phase contrast examination.

Inward current is carried by chloride ions

The absence of permeant cations in the external solution (except for 2 mM $CaCl_2$ and 1 mM $MgCl_2$) suggested that the inward current elicited by the hyperpolarization jumps was carried by chloride ions. Preliminary experiments in which external NMG^+ was replaced by Na^+ showed no change in the amplitude of the hyperpolarization-activated inward current.

Further evidence that this current was carried by Cl^- was obtained by analysing the reversal potential of either the tail currents corresponding to deactivation of the hyperpolarization-activated current, or of the instantaneous current–voltage relationship of activated channels. When E_{Cl} (the equilibrium potential for Cl^-) was 0 mV (external standard solution and internal solution 1, Table 1), the tail currents reversed about 0 mV, and deactivated rapidly at positive potentials (Fig. 3*A*). After the external isotonic replacement of 140 mM Cl^- by the less permeant anion, glutamate (E_{Cl} of +80 mV), tail currents did not reverse up to +60 mV (Fig. 3*B*) indicating that the inward current activated by hyperpolarization was carried by chloride ions. (Larger depolarizing pulses often caused deterioration of the membrane, thus impairing reliable current recordings.) The instantaneous current–voltage relationship was established using fast voltage ramps (0·3 s) between −100 and +60 mV at the end of a hyperpolarizing jump from 0 to −120 mV. The *I–V* curve established under symmetrical Cl^- concentrations presented a slight inward rectification (Fig. 3*C*).

Ion selectivity was investigated by replacing 90 mM of external chloride by equal concentrations of iodide, bromide or glutamate. As would be expected for chloride channels, the reversal potential of the instantaneous *I–V* curve was shifted to positive values when chloride was replaced by glutamate (E_{Cl} of +24 mV) (Fig. 3*C*). Analysis of the reversal potentials measured after iodide- or bromide-for-chloride substitutions indicated that the channels displayed a $Cl^- > Br^- > I^-$ > glutamate selectivity sequence (Fig. 3*C*). Note that iodide-for-chloride substitution resulted in a decrease of the overall current, indicating that iodide partially blocks the channel.

The voltage-activated Cl^- current did not require calcium ions for its activity

The chloride current amplitudes recorded with 10 mM EGTA in the internal solution (solution 2, Table 1) were similar to those recorded in the presence of 0·5 mM EGTA (solution 1, Table 1), as shown in Fig. 2. Omission of Ca^{2+} from the bath solution, in which 2 mM $CaCl_2$ was replaced by 2 mM $MgCl_2$, did not affect the amplitude of the relaxation (4 cells).

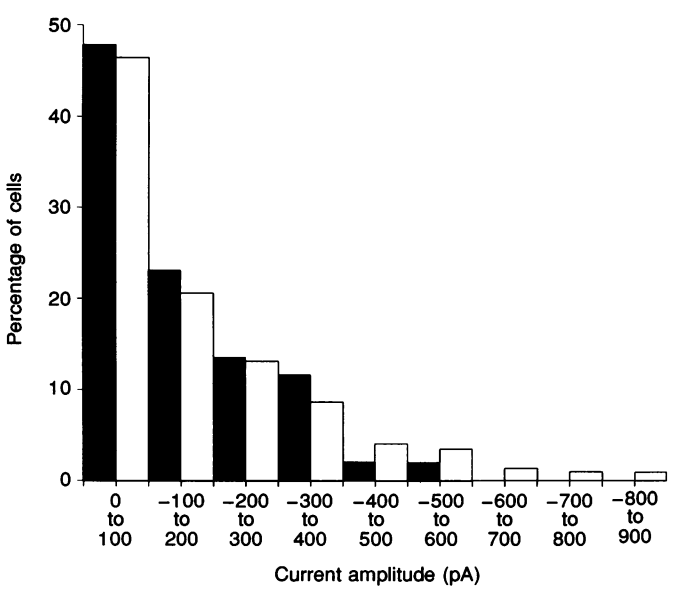

Figure 2. Current amplitude histogram

Histogram of current amplitudes recorded 20 s after a jump to −120 mV, from 230 T_{84} human adenocarcinoma cells using the high-chloride pipette solution containing 0·5 mM EGTA (solution 1, Table 1; □) and from fifty-two cells using the high-chloride pipette solution containing 10 mM EGTA (solution 2, Table 1; ■).

Pharmacological properties of the hyperpolarization-activated Cl^- current

The effects of organic and inorganic anionic inhibitors on $I_{Cl,hyp}$ were tested. External application of 1 mM DPC partly and reversibly inhibited by 47 ± 13% the Cl^- current measured in seven cells at −120 mV (Fig. 4*A* and *B*). This current was also sensitive to another standard chloride channel blocker, NPPB (Fig. 4*C*). At 50 μM, this blocker reduced the amplitude of the current activated at −120 mV by 26·8 ± 3·9% in four cells and at 100 μM, by 47·3 ± 4·9% also in four cells. Neither DPC nor NPPB induced any current at 0 mV.

External application of 1 mM DIDS had no effect at any potential tested.

Cadmium ions were found to be the most potent blockers. $CdCl_2$ (1 mM) caused a complete and reversible blockade ($n = 6$). The effects induced by 30 and 300 μM CdCl are illustrated in Fig. 4*D*. $CdCl_2$ (30 μM) reduced the amplitude of the relaxation recorded at −120 mV by 53·6 ± 5·4% in six cells, and 300 μM $CdCl_2$ blocked the current almost completely, by 92·9 ± 6·6% in thirteen cells.

Addition of 1 mM $BaCl_2$ to the bath solution had no effect on $I_{Cl,hyp}$.

Dependence of $I_{Cl,hyp}$ on the internal Cl^- concentration

When T_{84} cells were dialysed with the low-chloride internal solution (20 mM, solution 3, Table 1), only 13% of the cells displayed inward current relaxations, with amplitudes that did not exceed 15 pA for a voltage jump from 0 to −120 mV. This proportion rose to 60% when a higher internal chloride concentration was used (30 mM, solution 4, Table 1). Under these conditions, the amplitude of the current relaxation varied from cell to cell between 25 and 250 pA, in response to the same voltage jump. Figure 5 shows the current traces and *I–V* relationship obtained under symmetrical low-Cl^- conditions (30 mM external and internal Cl^- concentrations). Activation of the current

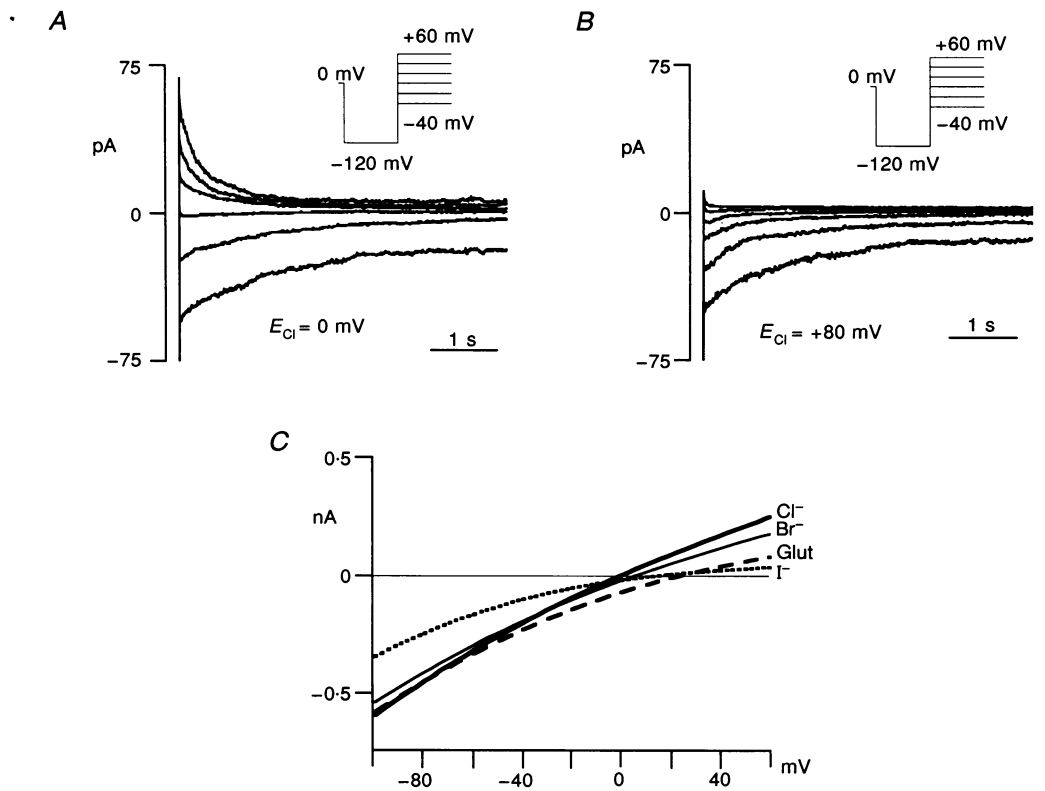

Figure 3. Reversal potentials of activated channels and ionic selectivity

A and *B*, tail currents corresponding to the deactivation of the conductance activated by a 10 s prehyperpolarizing jump to −120 mV, according to the voltage jump protocol described in the insets. *A*, tail currents recorded during repolarization (between −40 and +60 mV) in the standard external solution (E_{Cl} at 0). *B*, currents recorded after replacement of 140 mM chloride by 140 mM glutamate in the external solution (E_{Cl} at +80 mV). The tail currents reversed close to 0 mV in *A* and did not reverse up to +60 mV in *B*. *C*, instantaneous *I–V* relationships of activated channels in the presence of various extracellular anions. A fast voltage ramp (300 ms, to minimize deactivation effects) from −100 to + 60 mV was applied immediately after activation of the channels by a hyperpolarizing jump for 10 s to −120 mV. Ion selectivity was tested by replacing 90 mM of extracellular chloride with equal concentrations of bromide, iodide or glutamate (Glut).

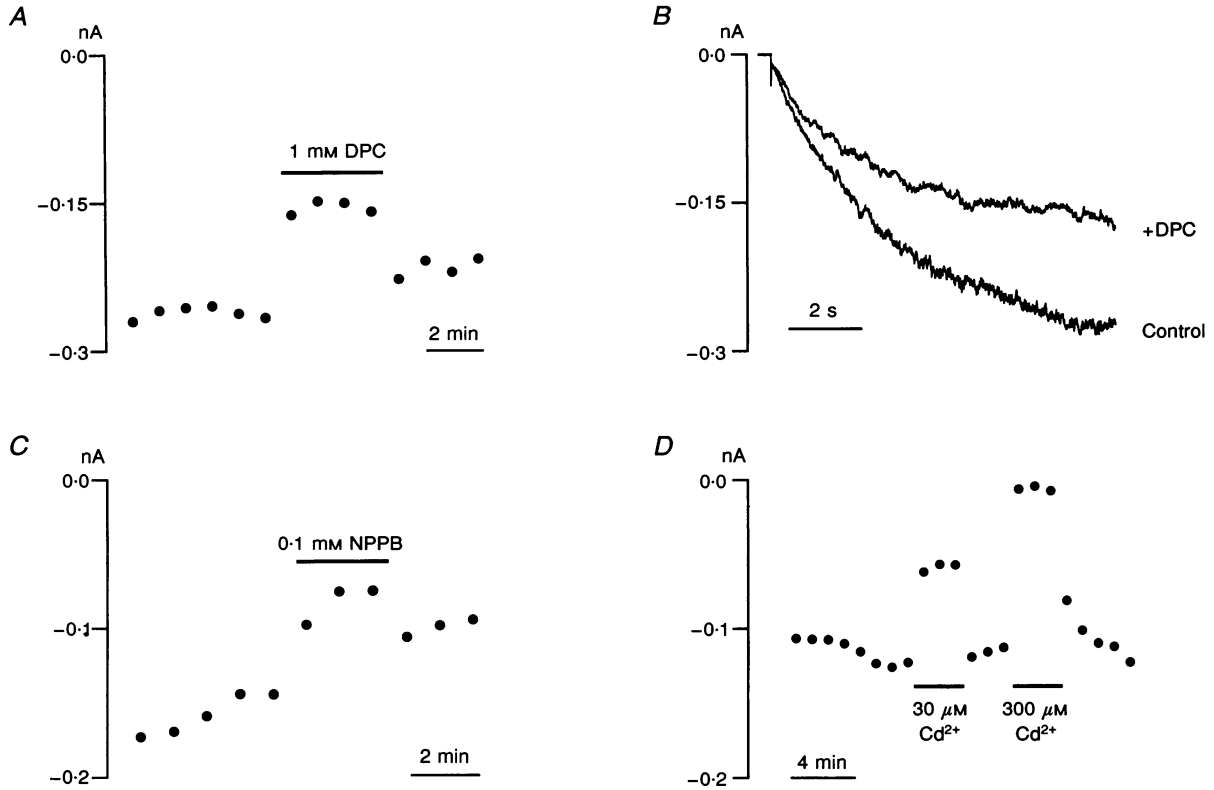

Figure 4. The inhibitory effects of DPC, NPPB and Cd^{2+}

A, plot of $I_{Cl,hyp}$ amplitude recorded during successive voltage jumps delivered every 60 s from 0 to −120 mV, in the absence and presence of 1 mM DPC. *B*, corresponding current traces recorded before and during the application of DPC. *C* and *D*, plots of $I_{Cl,hyp}$ amplitude recorded during regular voltage jumps applied from 0 to −120 mV in the absence and presence of 100 μM NPPB (*C*), or 30 and 300 μM Cd^{2+} (*D*).

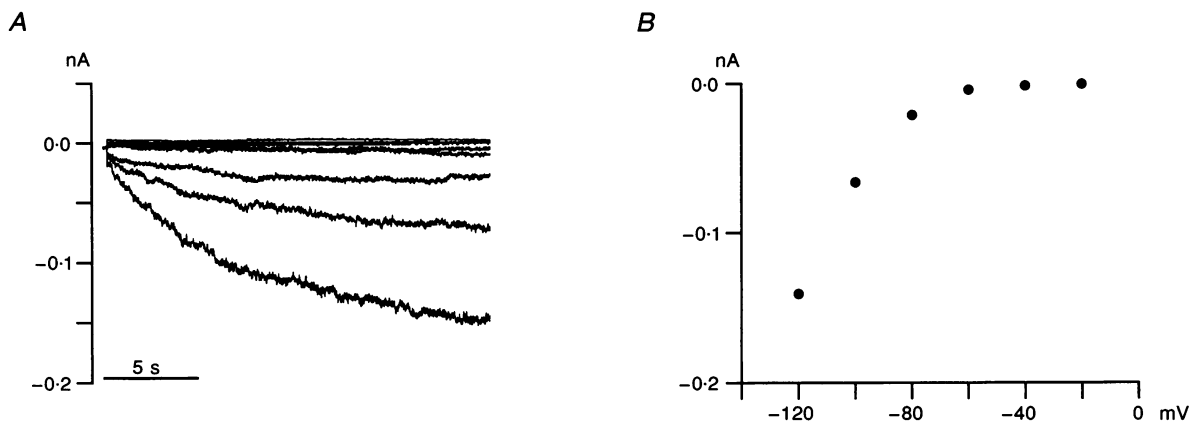

Figure 5. *I–V* curve of the current activated by hyperpolarization in the presence of symmetrical low-Cl^- solutions

A, current traces recorded during a series of voltage jumps described in Fig. 1*A*, in a cell dialysed with an internal solution containing 30 mM chloride and ATP (solution 1, Table 1). The external solution was the same as in Fig. 1 except that 116 mM NMGCl was replaced with 116 mM NMG glutamate. *B*, corresponding *I–V* curve of the current relaxation measured as the difference in current amplitudes measured between 10 s and 30 ms.

relaxation could be detected above −60 mV, i.e. 20 mV more negative compared with experiments performed with high-chloride pipette solutions. In addition, qualitative comparison of the kinetics of activation of $I_{Cl,hyp}$ in both experimental conditions showed that low internal concentrations were associated with slower kinetics. Since quantitative estimations of the time constants of activation could not be satisfactorily obtained from exponential fits, we simply took fractional current activation (F_a), defined as the ratio of current amplitudes at 4 s compared with 20 s, as an index of activation kinetics. At −120 mV, F_a was 0·48 ± 0·07 ($n = 10$) and 0·73 ± 0·05 ($n = 20$) for low- and high-chloride conditions, respectively.

Modulation of $I_{Cl,hyp}$ by cAMP and phorbol esters

External perfusion of 500 μM of the permeant analogue cptcAMP resulted in a decrease in the amplitude of the inward current relaxation (Fig. 6*A*). This effect was reversible upon washing out of the nucleotide when the application lasted for less than 5 min. The amplitude of the current recorded at −120 mV was decreased by 29·8 ± 10·9% in sixteen cells. This decrease displayed no voltage dependence (Fig. 6*B*, *C* and *D*). No effect was observed in the positive voltage range (Fig. 6*C* and *D*). Recording of the current at various potentials required longer applications of the cAMP analogue. Under these conditions, the inhibition of current amplitude was never fully reversible (Fig. 6*C*). In two of the cells studied, forskolin (20 μM) also induced small and slowly reversible decreases of this amplitude of 18 and 30%, respectively.

We investigated the possible modulatory effect of protein kinase C (PKC) by external perfusion of the PKC-stimulating phorbol ester PMA. Since phorbol esters have been reported to affect membrane conductances independently of PKC activation (Hockberger, Toselli,

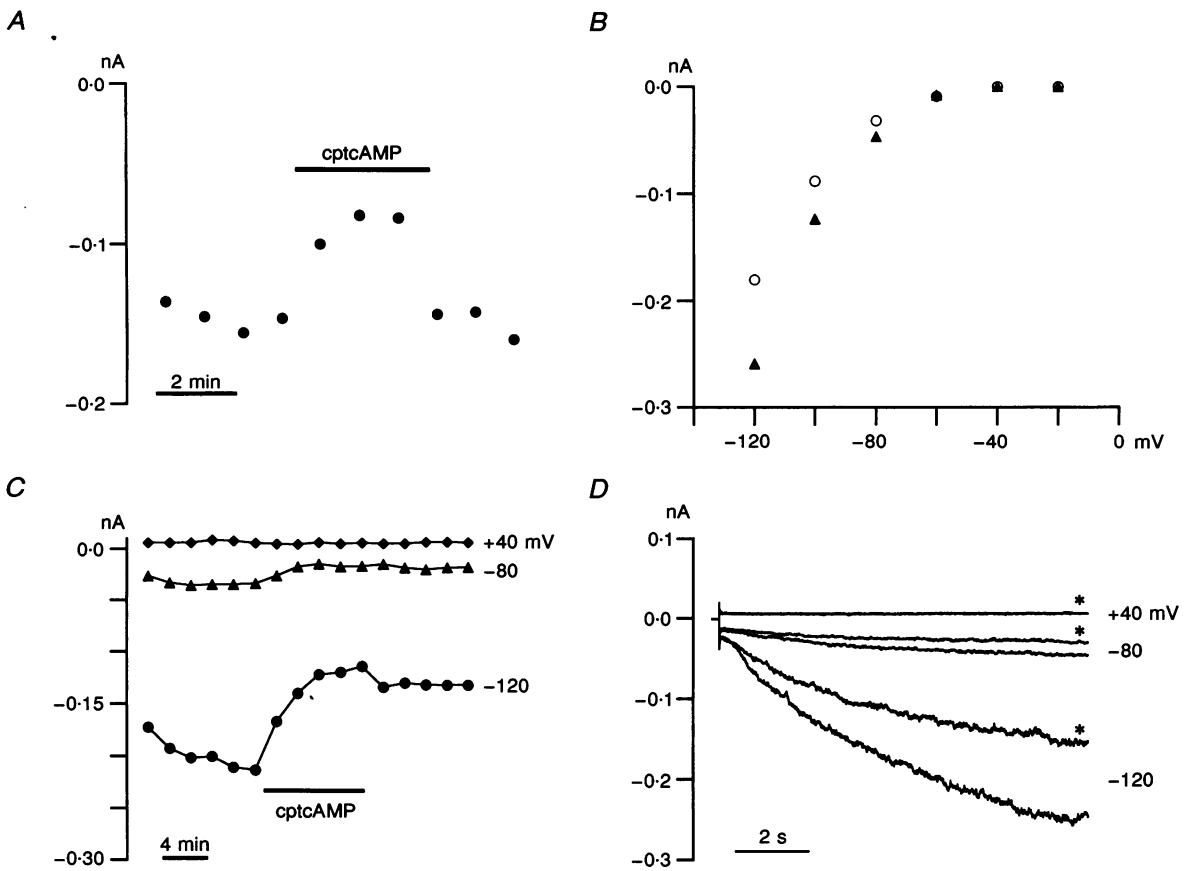

Figure 6. Inhibitory effect of cptcAMP

A, plot of $I_{Cl,hyp}$ amplitude recorded during successive voltage jumps from 0 to −120 mV in the absence and presence of 500 μM cptcAMP added to the external solution. cptcAMP was applied for 3 min and the inhibitory effect was reversible upon washing. *B*, *I–V* curves of current relaxation before (▲) and after (○) cell exposure to 500 μM cptcAMP. *C*, plot of $I_{Cl,hyp}$ amplitude recorded in a different cell during voltage jumps delivered alternately from 0 to −80, −120 and +40 mV in the absence and presence of cptcAMP (500 μM). cptcAMP was applied for 10 min. *D*, corresponding current traces recorded at −80, −120 and +40 mV before and during (*) cptcAMP application.

Swandulla, & Lux, 1989), we first tested the effect of 4αPMA, a phorbol ester analogue that is inactive with respect to PKC. 4αPMA (500 nM) produced no effect on $I_{Cl,hyp}$ in two cells, but in six others a slight decrease in the amplitude of the current (18·4 ± 4·9%) was detected. When 500 nM PMA was applied after 4αPMA, a more pronounced decrease in current amplitude was observed. Figure 7 illustrates an experiment on the same cell, in which 500 nM of 4αPMA induced a small decrease in the current amplitude, whereas 500 nM PMA induced clear irreversible inhibition. In eight cells pre-exposed to 4αPMA, application of 500 nM of PMA reduced current amplitude by 43·4 ± 16·1%. Figure 7*C* shows that the effect of PMA was voltage independent; in this cell the reductions of amplitude of $I_{Cl,hyp}$ at −120, −100 and −80 mV were not significantly different (60, 58 and 57%, respectively), and no effect was observed at +40 mV. This voltage independence was confirmed in four other cells (not shown).

Modulation of $I_{Cl,hyp}$ by internal ATP

To discriminate between the possible direct effects of cptcAMP and PMA on the one hand, and the effects due to phosphorylation mechanisms linked to the activation of their respective protein kinases on the other, we first tried to conduct the same experiments in the absence of intracellular ATP. However, without ATP in the pipette solution the activation kinetics and amplitude of the inward current rose sharply within the first 10 min of recording, as illustrated in Fig. 8*A* and *B*. Time scale expansion of the current traces illustrated in Fig. 8*B* showed that the increase in the current amplitude was not due to the development of an instantaneous inward current (Fig. 8*C*). Inward relaxation was clearly detectable at −40 mV, as shown in Fig. 9*A*, and the inward rectification was less pronounced than that observed in the presence of ATP (compare Fig. 9*B* and Fig. 1*C*). Replacement of external chloride by glutamate shifted the reversal potential of tail currents to more positive values, indicating that the overall current was still carried by Cl^-. These results suggest that either the gating properties of $I_{Cl,hyp}$ can be modulated by internal ATP, or that ATP blocked a separate voltage-activated chloride current. We tested this second possibility by examining the pharmacological properties of the overall activated current in the absence of ATP, and its dependence on the internal chloride concentration. The current was still blocked by 300 μM Cd^{2+} (Fig. 9*C*), partially

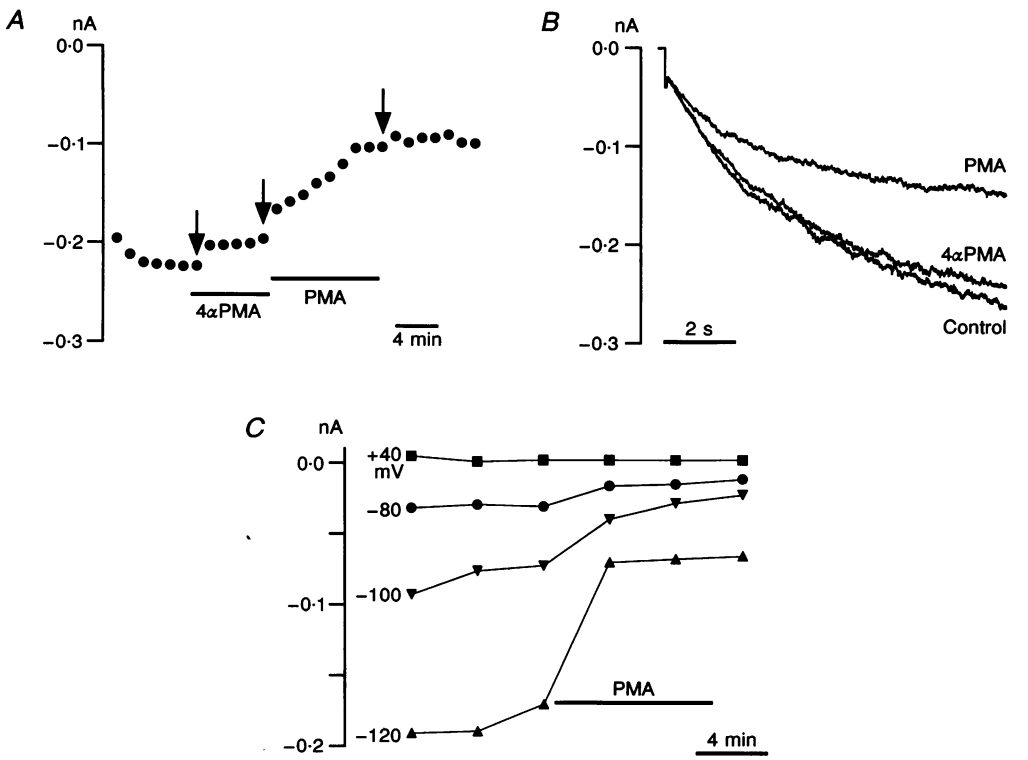

Figure 7. Inhibitory effect of the PKC-stimulating analogue PMA

A, plot of $I_{Cl,hyp}$ amplitude recorded during regularly delivered voltage jumps from 0 to −120 mV. The inactive phorbol ester (4αPMA) and the PKC-stimulating analogue PMA were applied successively, both at 500 nM. *B*, corresponding current traces recorded before and during application of 4αPMA and PMA. *C*, plot of $I_{Cl,hyp}$ amplitudes recorded during voltage jumps delivered alternately from 0 mV to −80, −100, −120 and +40 mV in the absence and presence of 500 nM PMA.

blocked by DPC and insensitive to the external application of DIDS or Ba^{2+}. The changes in the activation kinetics of $I_{Cl,hyp}$ observed in the absence of ATP in the pipette solution were also dependent on the internal chloride concentration. Omission of ATP from the solution containing 20 mM Cl^- (solution 3, Table 1) did not induce any voltage-activated current in eight cells which exhibited no current relaxation at the start of cell dialysis. In one cell which developed a small $I_{Cl,hyp}$ in response to the first voltage jump to −120 mV, omitting ATP from the pipette solution induced a gradual increase in the activation rate of the current, similar to that observed when the cells were dialysed with the high-Cl^-, ATP-free internal solution (solution 1, Table 1). Taken together, these results indicate that the gating properties of $I_{Cl,hyp}$ were indeed modulated by ATP.

Regulation of $I_{Cl,hyp}$ by phosphorylation mechanisms

Replacement of 3 mM ATP in the internal solution by an equivalent amount of the non-hydrolysable analogue AMPPNP induced the same changes in current amplitude and time course of current activation as those which occurred in the absence of ATP (4 cells), thus demonstrating that modulation of $I_{Cl,hyp}$ by ATP required a hydrolysis step.

We investigated the effects of various protein kinase inhibitors, to determine whether the regulation of $I_{Cl,hyp}$ involved the phosphorylation and activation of protein kinases under basal conditions. Addition of 20 μM of the cAMP-dependent protein kinase inhibitor to the pipette solution containing ATP (solution 1, Table 1) did not change either the amplitude of the inward chloride current or its activation kinetics. In contrast, the addition of a protein kinase C inhibitor (500 nM staurosporine or 200 nM calphostin C) induced the same changes as those observed in the absence of ATP (or in the presence of AMPPNP). Figure 10*A* illustrates the current traces recorded at −120 mV in a cell dialysed for 2 and 15 min with staurosporine and the ATP-containing internal solution (staurosporine was also added to the bath at the same concentration). A similar effect was observed for staurosporine in four out of four cells, and for calphostin C in four other cells studied with this inhibitor (not shown). The addition of DMSO alone (final concentration, 0·1%) to both the external and internal solutions did not change the amplitude or activation rate of $I_{Cl,hyp}$. In Fig. 10*B* 300 μM Cd^{2+} blocked the current that developed in the presence of staurosporine.

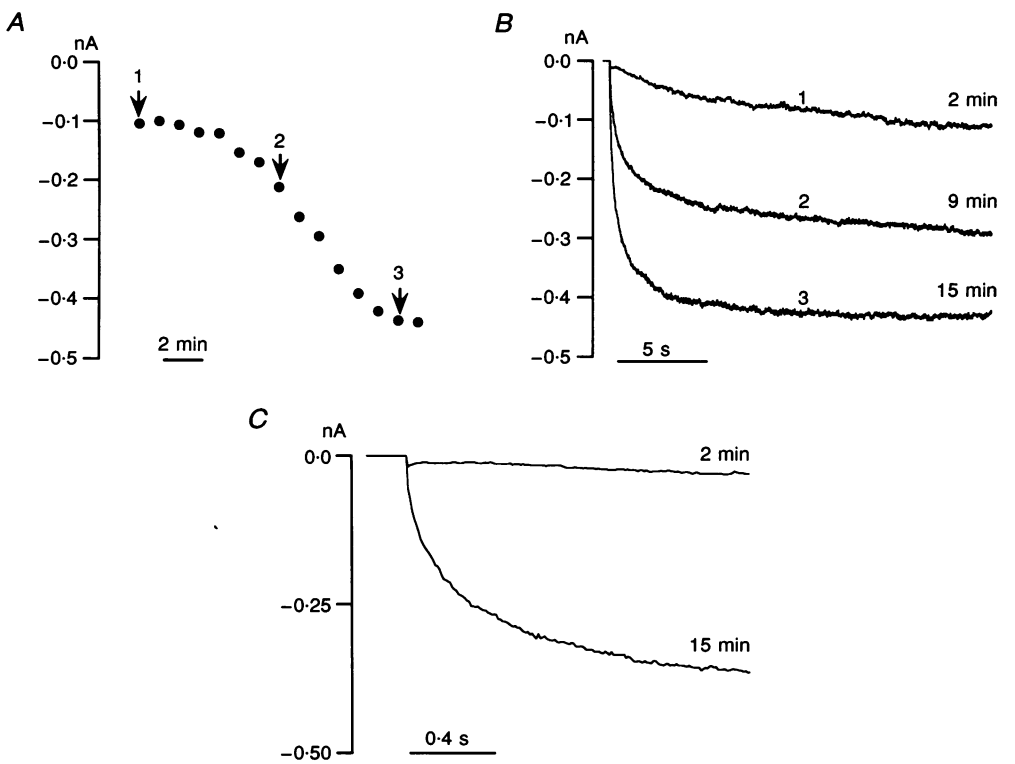

Figure 8. Effect of intracellular ATP depletion on $I_{Cl,hyp}$

A, evolution with time of the current amplitude recorded 20 s after a jump to −120 mV in a cell dialysed with the ATP-free high-chloride pipette solution (solution 1, Table 1). *B*, current traces recorded during 20 s voltage jumps, 2, 9 and 15 min after the start of whole-cell recording. *C*, part of the current traces illustrated in *B* on an expanded time scale, showing that the acceleration of the activation kinetics and the increase in current amplitude cannot be ascribed to the development of an instantaneous inward current.

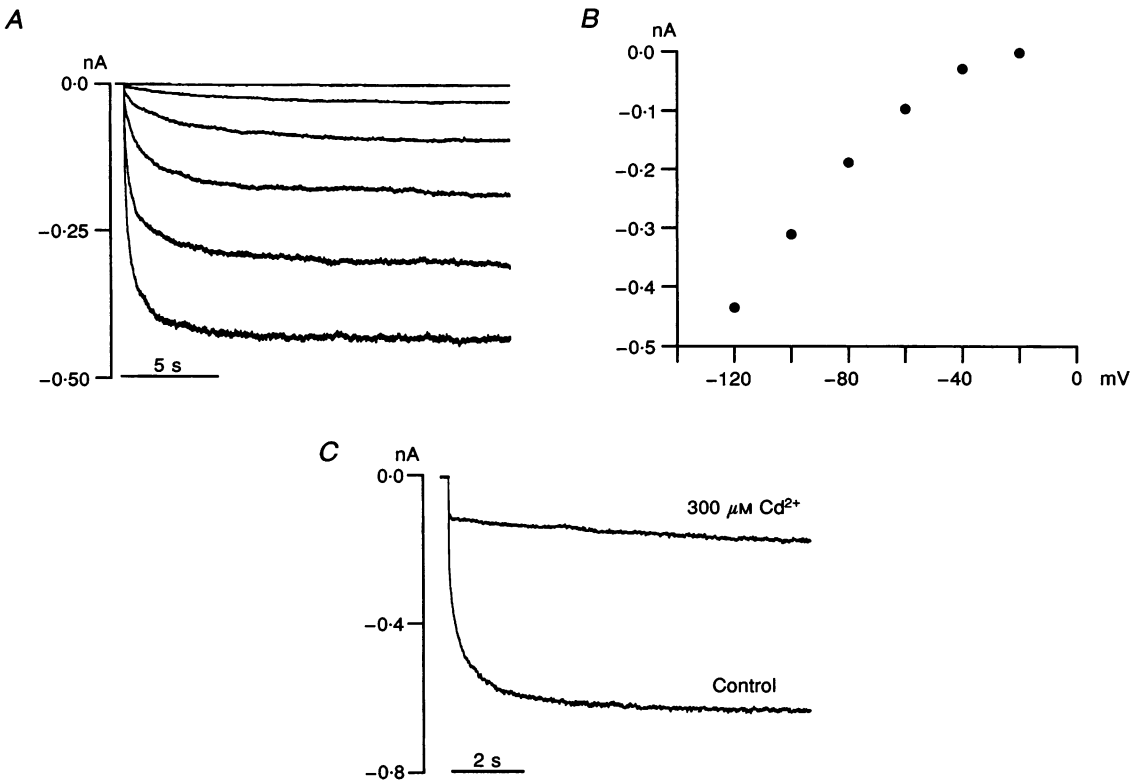

Figure 9. I–V curve and Cd^{2+} sensitivity of $I_{Cl,hyp}$ following intracellular ATP depletion

A, same cell as in Fig. 8. Superimposed current traces recorded during successive voltage jumps according to the voltage protocol described in Fig. 1*A*. *B*, corresponding I–V curve of the current amplitude measured at the end of the hyperpolarizing jumps. *C*, blocking by 300 μM Cd^{2+} of the chloride current recorded at −120 mV.

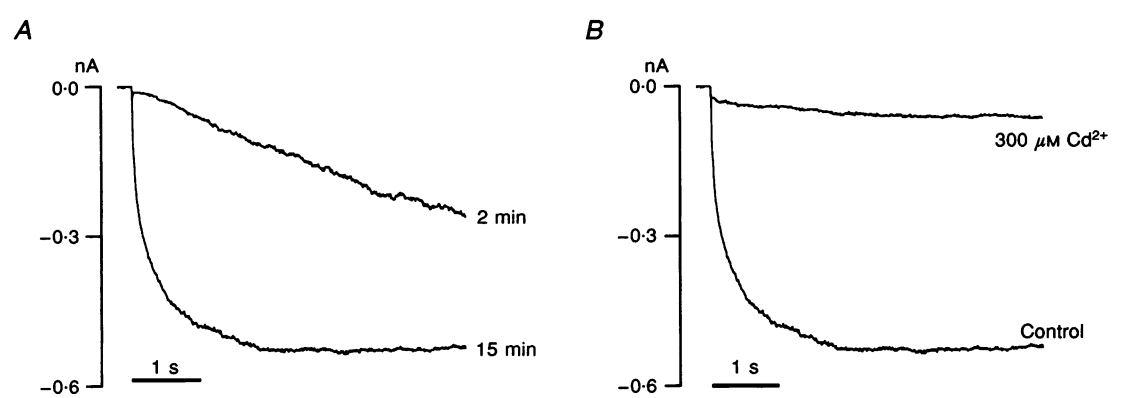

Figure 10. Effect of the PKC inhibitor staurosporine

A, current traces recorded at −120 mV, 2 and 15 min after the start of whole-cell recording using the high-chloride pipette solution containing 3 mM ATP and 500 nM staurosporine. *B*, same cell as in *A*. Current traces recorded before and during the application of 0·3 mM Cd^{2+}

DISCUSSION

This study demonstrates the existence of an inward current which is slowly activated by hyperpolarization in T_{84} cells. This current, which was recorded without Na^+ or K^+ in the external solution, is carried by Cl^-, as shown by the reversal potential of tail currents, and by its sensitivity to the intracellular chloride concentration. Although T_{84} cells have been used in many patch-clamp studies, the presence of a hyperpolarization-activated current has not been mentioned in any previous report, perhaps because of differences between the voltage jump protocol used in our study and the protocols used in other investigations. An inadequate amplitude or a too short duration of hyperpolarizing voltage jumps would impair the detection of current relaxation, at least for currents of moderate size.

Comparison with other hyperpolarization-activated chloride currents

Voltage-activated chloride currents have been found in several cell types, and have been characterized by their threshold potentials, activation kinetics and pharmacological sensitivity. Some of them require strong negative membrane potentials of more than −100 mV for activation, such as the currents recorded after the expression of phospholemman and ClC-2 proteins in the oocyte (Moorman, Palmer, John, Durieux & Jones, 1992; Thiemann *et al.* 1992). The threshold potential of $I_{Cl,hyp}$ determined from our results was close to −40 mV. This value lies within the range of the membrane potentials determined in T_{84} cells and isolated crypt cells, either in the resting state or after stimulation by secretagogues (Walters & Sepulveda, 1991 and included references), and suggests that the voltage-activated channels can be opened under physiological conditions.

As previously reported for *Aplysia* neurons (Chesnoy-Marchais, 1983), mandibular cells (Dinudom *et al.* 1993) and osteoblasts (Chesnoy-Marchais & Fritsch, 1994), we observed here that activation of the current was dependent on the internal chloride concentration. Relaxation currents were rarely detected when the internal solutions contained only 20 mM chloride ions, and at −120 mV their amplitude did not exceed a few picoamps. However, both the number of responsive cells (i.e. cells in which an inward current relaxation was detected) and the maximal amplitude of the current recorded at −120 mV rose markedly when the internal chloride concentration was increased by only 10 mM, from 20 to 30 mM. Under these conditions, the change in the magnitude of the relaxation was greater than expected for the relatively small increase in the driving force for chloride ions. The different thresholds and kinetics of activation of $I_{Cl,hyp}$ recorded under low or high symmetrical Cl^- conditions are reminiscent of the behaviour of the chloride conductance described in *Aplysia* neurons (Chesnoy-Marchais, 1983). This author proposed a scheme implying a binding site for internal chloride ions which could account for the sensitivity of the conductance to the intracellular chloride concentration. Whatever the mechanisms that might be implicated, the chloride concentration in resting T_{84} cells, estimated at about 50 mM (Huflejt, Blum, Miller, Moore & Machen, 1994), appears to be large enough to activate $I_{Cl,hyp}$.

A hyperpolarization-activated Cl^- current is sensitive to DPC, NPPB and Cd^{2+}, but insensitive to external DIDS and Ba^{2+}. The various voltage-activated chloride channels previously described can be partly distinguished by their pharmacological sensitivity to DIDS and cations. Some are sensitive to externally applied DIDS (Attali *et al.* 1993; Noulin & Joffre, 1993; Chesnoy-Marchais & Fritsch, 1994), while others are not (Chesnoy-Marchais, 1983; Lotshaw & Levitan, 1987; Thiemann *et al.* 1992; Komwatana *et al.* 1994; Kowdley *et al.* 1994), and some are inhibited by Ba^{2+} (Moorman *et al.* 1992; Attali *et al.* 1993; Kowdley *et al.* 1994), while others are not (Block & Moody, 1990; Chesnoy-Marchais & Fritsch, 1994). On the basis of these criteria, $I_{Cl,hyp}$ is distinct from the voltage-activated chloride current recorded in *Aplysia* neurons (Chesnoy-Marchais, 1983; Lotshaw & Levitan, 1987), rat osteoblasts (Chesnoy-Marchais & Fritsch, 1994), *Xenopus* oocytes (Kowdley *et al.* 1994), and granular duct cells from mouse mandibular glands (Komwatana *et al.* 1994) as well as from the currents activated after the expression in oocytes of the two small proteins phospholemman and the delayed rectifier K^+ channel protein IsK (Moorman *et al.* 1992; Attali *et al.* 1993). As predicted by the expression of ClC-2 mRNA, the anion selectivity and the pharmacological responses of $I_{Cl,hyp}$ to DIDS and DPC were similar to those of the ClC-2 chloride conductance (Tiemann *et al.* 1992), implying the presence in T_{84} cells of a functional ClC-2 protein.

The same sensitivities towards DPC, NPPB and DIDS were found for the CFTR chloride channel responsible for cAMP-stimulated chloride secretion in T_{84} cells (for review see Anderson *et al.* 1992). Therefore, these agents cannot be used to discriminate between the chloride ions which may flow out through the voltage-activated channels, and those leaving by the CFTR pathway. The sensitivity of $I_{Cl,hyp}$ to low Cd^{2+} concentrations, also found in other preparations (Madison *et al.* 1986; Chesnoy-Marchais & Fritsch, 1994), might help to answer this question.

Phosphorylation-dependent regulation of $I_{Cl,hyp}$

Little is known about the modulation of hyperpolarization-activated chloride currents by intracellular messengers and/or protein kinases. The inhibitory and voltage-dependent effects of cAMP in *Aplysia* neurons (Lotshaw & Levitan, 1987) have been ascribed to a shift in the voltage dependence of chloride conductance towards more negative potentials. It has also been proposed that cAMP modulates voltage-activated Cl^- conductance in Leydig cells (Noulin & Joffre, 1993). In the latter study, raising intracellular cAMP

increased the rate constants of current activation but did not cause a marked increase in the magnitude of the current in the negative voltage range. We observed that cAMP induced a small, voltage-independent inhibition of $I_{Cl,hyp}$. This inhibition cannot be explained by an increase in an outward current, since cAMP had no effect above the activation threshold. In the course of our study we rarely observed activation of the CFTR chloride current by cAMP. Application of cptcAMP resulted in a small increase in membrane conductance on each side of E_{Cl} in only three out of sixteen cells. This small proportion was probably linked to the fact that our experiments were performed at room temperature and on individual cells. Recently, Overholt, Saulino, Drumm & Harvey (1995) reported that cAMP-stimulated CFTR current could be observed in only 25% of the T_{84} cells studied at room temperature, whereas an earlier study (Cliff & Frizzell, 1990) showed that the responsiveness of the CFTR chloride channel improved when cells were maintained at 37 °C and studied as parts of small islands.

$I_{Cl,hyp}$ was also inhibited by the PKC-activating phorbol ester PMA. A similar inhibition has been described for PKC stimulation in hippocampal pyramidal cells. High doses of phorbol ester (10 μM 4β-phorbol 12,13-dibutyrate) completely blocked the Cd^{2+}-sensitive and voltage-activated chloride current present in these cells (Madison *et al.* 1986).

Depletion of intracellular ATP increased the activation kinetics and amplitude of the inward current relaxation. The question, therefore, arises as to whether these changes were due to modulation by ATP of the gating properties of one class of hyperpolarization-activated chloride channels, or to the gradual release of an ATP-induced inhibitory effect on a separate class of chloride channels. Several arguments support the first alternative. Firstly, there was a good correlation between the changes in current amplitude induced in the absence of ATP and the detection of $I_{Cl,hyp}$ at the beginning of whole-cell recording. Secondly, the pharmacological responses to DPC, DIDS, Cd^{2+} and Ba^{2+} were the same, both in the absence and presence of ATP. Therefore, the hyperpolarization-activated chloride current is indeed regulated by ATP in T_{84} cells, unlike mandibular gland cells in which the absence of such regulation of the voltage-activated chloride current was reported (Komwatana *et al.* 1994).

The fact that the inhibitory effect of ATP on $I_{Cl,hyp}$ could not be reproduced by the non-hydrolysable analogue AMPPNP indicated that the changes in $I_{Cl,hyp}$ gating properties were due to dephosphorylation of the channel protein itself, or of some associated regulatory protein. The regulation of $I_{Cl,hyp}$ by ATP can thus be distinguished from the direct inhibition by cytosolic ATP of different chloride channels described in rabbit cortical collecting duct cells and sheep tracheal epithelium (Alton, Manning, Schlatter, Gedds & Williams, 1991; Superdock, Snyders & Breyer, 1993).

The changes in $I_{Cl,hyp}$ gating properties that followed dephosphorylation were reproduced by applying the two PKC inhibitors staurosporine and calphostin C. This suggested that in resting cells, the voltage-activated chloride channels could be tonically inhibited by a phosphorylation-dependent pathway involving PKC. These results strengthen the conclusion drawn from the PMA experiments, i.e. that the activation of PKC modulates $I_{Cl,hyp}$, and further implies that PKC might be constitutively active in T_{84} cells. Although not yet demonstrated in these cells, recent studies on intestinal cells *in situ* have shown that an active form of PKC associated with cytoskeletal elements is present at some stages of differentiation (Saxon, Zhao & Black, 1994). Induction of channel activity in resting cells by PKC inhibitors has already been observed for different types of chloride channels in other preparations, such as vascular smooth muscle cells and aortic endothelial cells (Kokubun, Saigusa & Tamura, 1991; Groshner & Kukovetz, 1992). In contrast to PKC, PKA apparently did not participate in the regulation of $I_{Cl,hyp}$ in the resting state, as indicated by the absence of effect of the PKA inhibitor. However, the activation of PKA may have a regulatory effect, as shown by the inhibitory effects of cptcAMP and forskolin.

The wide range of current amplitudes recorded in the presence of physiological concentrations of ATP suggests that cellular mediators other than PKC could be involved. It is also possible that this wide range depends on the differential expression of $I_{Cl,hyp}$, according to the proliferation and/or differentiation state of the cell. This hypothesis is supported by previous observations showing that expression of the hyperpolarization-activated chloride currents is suppressed during maturation of the frog oocyte (Taglietti *et al.* 1984) and is linked to the cell cycle in ascidian embryos (Block & Moody, 1990). Cell-to-cell variability in the expression of other types of chloride current has also been described in unstimulated T_{84} cells (Valverde, Mintenig & Sepulveda, 1994) while expression of the Ca^{2+}-dependent chloride current has been shown to depend on the growth state of T_{84} cells, as this current is only present in subconfluent cells (Anderson & Welsh, 1991).

Possible roles for $I_{Cl,hyp}$ in T_{84} cells

The presence of ClC-2 mRNA in many epithelial and non-epithelial tissues suggests that the ClC-2 protein has a housekeeping function. However, the strong cell negativity necessary to open the ClC-2 channel (in excess of −100 mV) in the oocyte expression system raises doubts about the activation of this channel *in vivo*. Nevertheless, exposure of oocytes to hypotonic solutions activated the ClC-2-related Cl^- current in the physiological voltage range, leading to the proposal that ClC-2 channels may help to regulate cell volume (Pusch & Jentsch, 1994). If the protein responsible for $I_{Cl,hyp}$ in T_{84} cells is encoded by the ClC-2 gene, one of its functions may be to contribute to cell volume regulation.

The present results demonstrating that $I_{Cl,hyp}$ is activated at physiological membrane potentials indicate that it may have other functions, such as participating in the regulation of the cell membrane potential. A second attractive hypothesis is to consider that $I_{Cl,hyp}$ may prevent an excessive increase in the intracellular chloride concentration and be involved in Cl^- secretion, either under basal conditions or after agonist stimulation. The apical pathway for chloride secretion in response to Ca^{2+}-mobilizing hormones in T_{84} cells has not yet been well defined. Assuming that the voltage-activated chloride channels are located in the apical membrane, they might constitute a possible exit pathway, since the cell negativity needed to open these channels can easily be reached after stimulating the cells with agonists that raise intracellular calcium (Walters & Sepulveda, 1991 and included references). $I_{Cl,hyp}$ may contribute to the apical DIDS-insensitive chloride pathway activated by carbachol in T_{84} cells (McEwan, Brown, Hirst & Simmons, 1993; McEwan, Hirst & Simmons, 1994). However, the negative effect that should result from the concomitant activation of PKC would probably limit this potential exit pathway. In addition, the sensitivity of $I_{Cl,hyp}$ to PKC inhibitors also sheds light on the events responsible for the enhancement of carbachol-stimulated Cl^- secretion in the presence of these inhibitors (Lindeman & Chase, 1992). Our data suggest that the resulting increase in $I_{Cl,hyp}$ amplitude contributes to the overall induced Cl^- secretion. However, the contribution of $I_{Cl,hyp}$, if any, to this secretion remains hypothetical since the properties of the chloride current have only been examined in isolated cells. Further studies are needed to determine whether hyperpolarization-activated chloride channels are present in the apical membrane of differentiated and polarized T_{84} cells.

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Acknowledgements

This work was supported by the Institut National de la Santé et de la Recherche Médicale and the Association Française de Lutte contre la Mucoviscidose.

Received 6 March 1995; accepted 6 July 1995.