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# Activation by Hyperpolarization and Atypical Osmosensitivity of a $Cl^-$ Current in Rat Osteoblastic Cells

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**Abstract.** During whole-cell recording of rat osteoblastic cells with high-$Cl^-$ internal solutions, 10 sec hyperpolarizing jumps from 0 mV induce a slow inward current relaxation, which is shown to be carried by hyperpolarization-activated $Cl^-$ channels. This relaxation increases and becomes faster with stronger hyperpolarizations. It is insensitive to $Cs^+$ ions but is blocked in a voltage-dependent manner by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) 1 mM and is reduced by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) 0.1 mM. $Cd^{2+}$ ions are potent blockers of this current, blocking completely above 300 μM. The amplitude of the $Cl^-$ current activated by a given hyperpolarization increases during the first 10–20 min of whole-cell recording. This evolution and the fact that some recently cloned $Cl^-$ channels have been reported to be activated both by hyperpolarization and by external hyposmolarity led us to investigate the effects of external osmolarity. Reducing the external osmolarity induces a large $Cl^-$ current. However, this hyposmolarity-induced $Cl^-$ current and the hyperpolarization-activated $Cl^-$ current are shown to be distinct; 1,9-dideoxy forskolin selectively blocks the hyposmolarity-activated current. We show that the hyperpolarization-activated $Cl^-$ current is osmosensitive, but in an unusual way: it is reduced by external hyposmolarity and is increased by external hyperosmolarity. Furthermore, these modulations are more pronounced for small hyperpolarizations. The osmosensitivity of the hyperpolarization-activated $Cl^-$ current suggests a mechanosensitivity (activation by positive external pressure) that is likely to be physiologically important to bone cells.

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**Key words:** $Cl^-$ current — Hyperpolarization-activated — Osmosensitivity — Mechanosensitivity — Osteoblast

## Introduction

In a previous study of voltage-gated $K^+$ currents of rat osteoblastic cells in the whole-cell configuration of the patch clamp technique [12], we noticed that during the first minutes of the recording with high-$Cl^-$ internal solutions some inward current developed at negative membrane potentials, without any correlated current change at 0 mV (chloride equilibrium potential close to 0 mV, potassium equilibrium potential close to −80 mV). To study $K^+$ currents properly, we had to prevent the development of this current, or to wait for its spontaneous disappearance.

The present paper describes this current which appeared to be a $Cl^-$ current activated by hyperpolarization. This type of current has been characterized in *Aplysia* neurons from intracellular voltage-clamp recordings [7, 8]. Single channel recordings later indicated that it was carried by two-state small conductance $Cl^-$ channels [9], similar to those of *Torpedo* electric organ studied after fusion of membrane vesicles with artificial phospholipid bilayers (*see ref.* 37, for review). In a few other cell types, hyperpolarization-activated $Cl^-$ currents have then been described, in particular in some vertebrate neurons [34, 48], in freshly isolated amphibian oocytes (without any particular mRNA injection), at certain stages of maturation [42, 50], and in ascidian embryos during cell division[4]. Single channel recordings from collecting duct basolateral membrane have also revealed two conductance state $Cl^-$ channels showing slow activation by hyperpolarization [45]. More recently, a multiconductance $Cl^-$

channel, usually studied in excised inside-out patches where it shows a bell-shaped voltage dependence, has been observed in cell-attached recordings from lymphocytes at $\geq 32°C$; under these conditions, it was shown to be activated by hyperpolarization in the physiological voltage range [41].

The *Torpedo* $Cl^-$ channel, named ClCO, has now been cloned and expressed in *Xenopus* oocytes [3, 30]. Two other $Cl^-$ channels which have been cloned recently, also appeared to be slowly activated by hyperpolarization when expressed in *Xenopus* oocytes: a channel homologous to ClCO, ClC2 [51], and a protein of different structure, the myocardium phospholemman [38].

In *Xenopus* oocytes injected with mRNA coding for ClC2, it has been shown that reducing the external osmolarity increases the total $Cl^-$ conductance and alters the kinetic properties of the hyperpolarization-activated current relaxation, and it has been concluded that the channel ClC2 itself can be activated by hyposmolarity [25].

It is known that many cell types respond to a decrease of the external osmolarity by an increase in $Cl^-$ conductance (*see ref.* 29 for a review of earliest studies; *see refs.* 5, 17, 21, 32, 33, 35, 49, 52, 54, 57 for whole-cell studies; *see refs.* 2, 14, 18 for single channel studies). In several cases, strong depolarizing jumps (above +50 mV) were shown to induce inactivation of the hyposmolarity-induced $Cl^-$ current [5, 32, 35, 49, 54], and in some cases, expressions like "activation at hyperpolarizing voltages" have been used to describe the reactivation of the current at a negative voltage after a strong depolarizing inactivating prepulse. However, none of the naturally expressed hyposmolarity-induced $Cl^-$ conductance was shown to be activated by hyperpolarization from a holding potential $\leq 0$ mV. ClC2 thus appears to be unique in being activated both by hyperpolarization and by external hyposmolarity.

The wide distribution of ClC2 indicates that hyperpolarization-activated $Cl^-$ channels are likely to be present in most cell types, but there are still very few studies of this type of channel in vertebrate cells. In the present study we found experimental conditions enabling the reproducible recording of a current activated by hyperpolarization in rat osteoblastic cells. We show that this current is carried by $Cl^-$ ions and study its pharmacological properties. In addition, we test whether this $Cl^-$ channel, activated by hyperpolarization from 0 mV, can also be activated by external hyposmolarity.

## Materials and Methods

The experiments were performed at room temperature on primary cultures of newborn rat osteoblastic cells, using the whole-cell configuration of the patch clamp technique.

### Cell Preparation

Osteoblastic cells were isolated from newborn rat calvaria as described in refs. [24] and [55]. Freshly excised calvaria were dissected out under a microscope: only the central part of parietal bones was used and both the inner and outer periostea were carefully stripped away to eliminate chondrocytes, suture cells and periosteal progenitor cells. Dissected bones were incubated at 37°C during two sequential 10 min periods in a $Ca^{2+}$-$Mg^{2+}$-free Earle's solution containing 0.5 mg trypsin/ml (Worthington) and 4 mM EDTA. Eighty percent of the cells thus isolated were mature osteoblasts [24, 55]. These cells were harvested, washed and seeded at about 10,000 cells/dish (35 mm Falcon) in BGJ medium (Flow Laboratories) supplemented with 10% fetal calf serum (Flow Laboratories), fungizone (2.5 µg/ml), penicillin (100 iu/ml) and streptomycin (50 µg/ml). Experiments were performed on single large polygonal cells from day 4 to day 6 in primary culture. Fusiform cells were avoided, as well as cells grouped in clusters.

### Solutions

Table 1 gives the composition of the main solutions used.

A stock solution of niflumic acid was prepared at 100 mM in dimethylsulfoxide (DMSO) and was then diluted 5,000 times in the external solution. Diphenylamine-2-carboxylate (DPC) was first diluted in hot ethanol at 500 mM; this solution was then immediately diluted 500 times in the external solution. A stock solution of NPPB was prepared at 50 mM in ethanol. A stock solution of 1,9-dideoxy forskolin (DDFSK, Calbiochem) was prepared at 16 mM in ethanol.

In all the experiments in which one of those compounds was used, all the external solutions successively applied on the cell by the fast perfusion system (*see below*) contained the same solvent dilution, so that the effects described cannot be due to the solvent itself.

### Perfusion System

A four-barrel fast perfusion system (made of glass and Teflon tubing, each barrel being connected to a glass syringe via a Teflon tap) was used for rapid application of the external solutions to be tested. One of the barrels was filled with the control external solution, the others with the test solutions. The recorded cell was continuously perfused with one of these barrels (solutions flowing by gravity), and the barrels were moved laterally to apply the desired solution onto the cell.

In addition, we also slowly perfused the whole culture dish either with the Na-5 K external solution, in the case of experiments performed in $K^+$-containing solutions, or with the Na-0 K solution, in the case of experiments performed with $K^+$-free solutions. We avoided perfusing the dish with sucrose-containing solutions, since this made local sucrose suppression by the fast perfusion system difficult.

### Recording and Analysis

Patch clamp micropipettes were made from hard-glass (Kimax 51); the shank of each pipette was covered with Sylgard and the tip was fire-polished. The resistance of these electrodes filled with high-$Cl^-$ internal solutions was between 3 and 6 MΩ. The cells were voltage-clamped by an EPC7 List amplifier, controlled by a TANDON 38620 computer, via a Cambridge Electronic Design (CED) 1401 interface, using CED patch and 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

**Table 1.** Composition (in mM) of the main solutions used

| External solutions | NaCl | or | Substitute | KCl | Sucrose |
|---|---|---|---|---|---|
| Na-5 K | 140 | | | 5 | 0 |
| NMG-5 K | 0 | | 140 NMG-Cl | 5 | 0 |
| Na-0 K | 145 | | | 0 | 0 |
| Na cyclamate-0 K | 0 | | 143 Na cyclamate | 0 | 0 |
| 105 Na-70 sucrose | 105 | | | 0 | 70 |
| 105 Na-0 sucrose | 105 | | | 0 | 0 |
| Na + sucrose | 145 | | | 0 | 42 |
| **Internal solutions** | **KCl** | | **K glutamate** | **CsCl** | **Cs glutamate** |
| High-$Cl^-$ $K^+$ | 140 | | 0 | 0 | 0 |
| Low-$Cl^-$ $K^+$ | 11 | | 127 | 0 | 0 |
| High-$Cl^-$ $Cs^+$ | 0 | | 0 | 143 | 0 |
| Low-$Cl^-$ $Cs^+$ | 0 | | 0 | 12 | 133 |

NMG: *N*-methyl-*D*-glucamine. HEPES: *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid. EGTA: ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid. All these external solutions also contained 10 mM HEPES buffered at pH 7.4 with NaOH or NMG (in the case of the NMG-5 K solution), 2 mM $CaCl_2$ and 1 mM $MgCl_2$. The osmolarity of the five external solutions was 290 mOsm. The 105 Na-0 sucrose was hypotonic (225 mOsm), whereas the Na + sucrose solution was hypertonic. All the internal solutions also contained 0.1 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES. Unless otherwise mentioned, they were always supplemented with 3 mM ATP-$Na_2$ and their final pH was adjusted to 7.3 using either KOH ($K^+$-containing solutions) or NaOH or CsOH ($K^+$-free solutions). The final osmolarity of these solutions was 280 mOsm (10 mOsm less than the external solution, which was necessary to avoid the rapid swelling of the cell occuring with internal solutions of higher tonicity). The osmolarity of all solutions was measured using a vapor pressure osmometer (Wescor 5500).

bridge, and junction potentials between the pipette solution and the bath solution were measured and corrected for.

When recording the currents in $K^+$-containing solutions, we usually applied 10 sec jumps from 0 to −100 mV every 20 sec. In the present study, under these conditions, the current recorded at 0 mV (chloride equilibrium potential) was stable with time after beginning of whole-cell recording. Cells showing a spontaneous progressive $K^+$ current increase during cell dialysis, $I_{K2}$ [12], were rarely found and were not used for the present study.

The voltage-jump protocol used in a given experiment was continuously repeated to follow the spontaneous evolution of the current studied. For example, the *I–V* curve protocol used in $K^+$-free solutions consisted of a series of 10 sec hyperpolarizing jumps of increasing amplitude each separated by a 10 sec (or 15 sec, in a few experiments) period at 0 mV; at the end of a series, a new series was automatically reapplied from 0 mV (pulse-sampling mode of CED software).

"Leak" and capacitive currents were not subtracted off-line. The optimum settings of the whole-cell capacitance cancellation of the EPC7 were used. However, due to the large surface of the cell, very flat and spread on the bottom of the dish, a slow capacitive component usually persisted (cell capacitance > 100 pF). The series resistance was between 6 and 12 MΩ when using the high-$Cl^-$ internal solutions. Measurement of the amplitude of the hyperpolarization-activated relaxation was performed by exponential fit of the relaxation, using the Strathclyde Electrophysiological Software (J. Dempster) VCAN program, which allows automatic rapid fits of series of traces. The data sampled by CED software were first transformed into a file compatible with the VCAN program (using a conversion program written in the Laboratoire de Neurobiologie by B. Martin). Fits were then performed on the portion of the current trace where the residual capacitive current was clearly zero (from 30–50 msec after the onset of the hyperpolarizing jump). The parameters automatically found by the program were considered as reliable when the fit and the original trace were superimposed up to the end of the voltage jump and when the error given for each parameter did not exceed 10% of the parameter value.

## Results

### Cell Dialysis with High-$Cl^-$ Internal Solutions Reveals an Inward Current Relaxation Activated by Hyperpolarization from 0 to −100 mV

Within the first minutes of whole-cell recording with the high-$Cl^-$ $K^+$ internal solution and the Na-5 K external solution (chloride equilibrium potential, $E_{Cl}$, close to 0 mV), voltage jumps between 0 and −100 mV (of 10 sec duration, regularly applied every 20 sec) revealed the progressive development of an inward current at −100 mV, not correlated with any current change at 0 mV. As shown by Fig. 1*A*, after several minutes of whole-cell recording, the voltage jump to −100 mV induced an increase in inward current occurring on a time scale of a few seconds (trace *2*), whereas at the beginning of the recording, the same voltage jump induced either no such relaxation or a relaxation of much smaller amplitude (trace *1*).

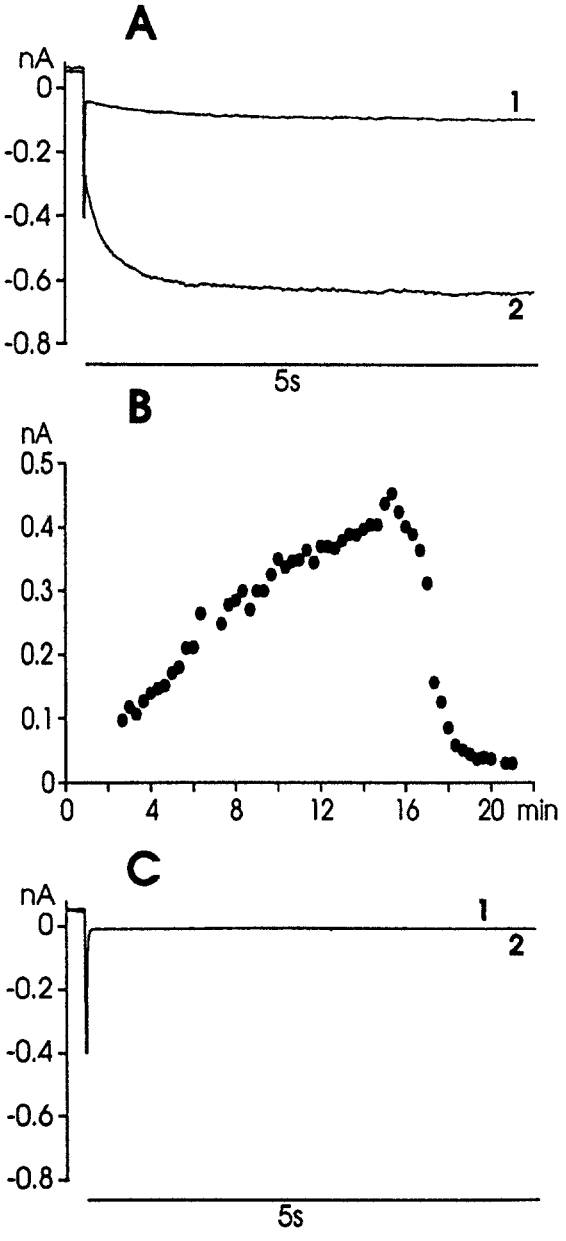


**Fig. 1.** Progressive development of an inward relaxation slowly activated by hyperpolarization during cell dialysis with a high-$Cl^-$ solution. Na-5 K external solution. (*A*) Current traces recorded during voltage jumps from 0 to $-100$ mV (regularly applied every 20 sec) after 2 min (*1*) or 14 min (*2*) of whole-cell recording with the high-$Cl^-$ $K^+$ internal solution. (*B*) Evolution with time after beginning of whole-cell recording (time 0) of the amplitude of the inward relaxation recorded during successive jumps from 0 to $-100$ mV. Same cell as *A*. (*C*) Current traces recorded in another cell, using the same voltage jump protocol but the low-$Cl^-$ $K^+$ internal solution, at the beginning of the recording (*1*) and after 14 min (2); no slow inward relaxation was ever observed during jumps from 0 to $-100$ mV.

In the experiment illustrated, the relaxation increased during the first 15 min of intracellular dialysis and voltage-jump stimulation, then decreased and disappeared after 20 min. The evolution with time of the amplitude of this relaxation is shown in Fig. 1*B*. Results qualitatively similar to those illustrated were obtained in several tens of experiments. However, the precise timecourse of the evolution of the relaxation varied from one experiment to another. In many cases, after the initial period of relaxation increase, there was a period of stability of a few minutes duration, before the final disappearance of the relaxation. This period was selected for the experiments performed in $K^+$-containing solutions.

When using the low-$Cl^-$ $K^+$ internal solution (instead of the high-$Cl^-$ $K^+$ internal solution) and the Na-5 K external solution ($E_{Cl}$ close to $-60$ mV), the current trace recorded during regular application of voltage jumps between 0 and $-100$ mV remained perfectly stable for tens of minutes (except in a few experiments where we observed the spontaneous development of a previously described $K^+$ current, $I_{K2}$, clearly detectable at 0 mV [12]). With these solutions, no inward current relaxation was ever detected at $-100$ mV (14 experiments). The stability of the current trace recorded under such ionic conditions is illustrated by the current traces of Fig. 1*C*.

## The Current Activated by Hyperpolarization is Carried by Chloride Ions

The timecourse of the inward relaxation recorded at $-100$ mV with the high-$Cl^-$ $K^+$ internal solution and the complete absence of a similar relaxation in the low-$Cl^-$ $K^+$ internal solution were reminiscent of the properties of the hyperpolarization-activated $Cl^-$ current described in *Aplysia* neurons [8]. To confirm that the current relaxation described by Fig. 1 was carried by chloride ions, several types of experiment were performed.

First, complete substitution of external $Na^+$ ions by a large cation, *N*-methyl-D-glucamine (NMG; two experiments), did not affect this relaxation (*not shown*) and complete removal of $K^+$ ions did not prevent its development (*see below*). Furthermore, the addition of 1–3 mM CsCl to the external solution (seven experiments in conditions of Fig. 1*A*, two experiments with the Na-0 K external solution and high-$Cl^-$ $Cs^+$ internal solution) did not affect the hyperpolarization-activated current of osteoblasts, whereas it is known to block the nonselective cationic current activated by hyperpolarization in some other cell types ($I_f$ in cardiac cells [19], $I_Q$ or $I_h$ in neurons (*see ref.* 36 and included refs)). These results indicate that the current studied is not carried by $Na^+$ or $K^+$ ions.

That this current is carried by $Cl^-$ ions was demonstrated more directly using the voltage-jump protocol of Fig. 2*A* (hyperpolarization from 0 to $-100$ mV fol-

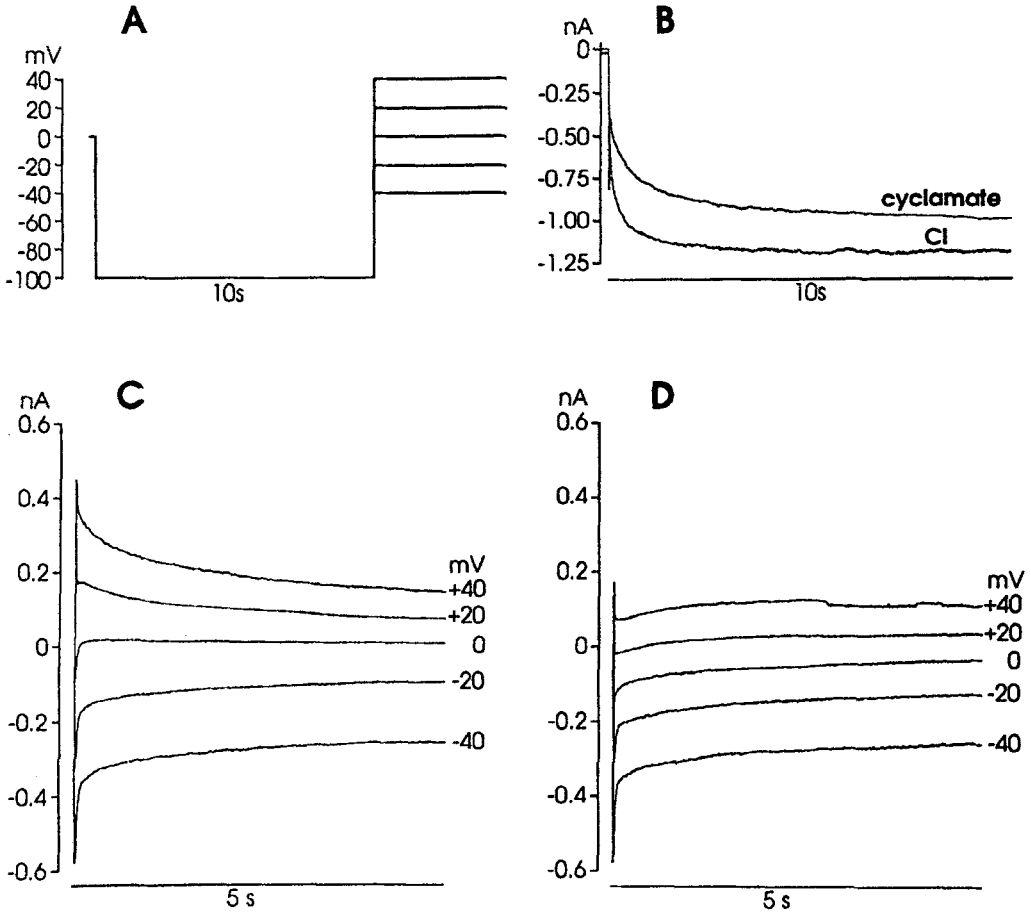


**Fig. 2.** $Cl^-$-sensitivity of the reversal potential of the tail current corresponding to the deactivation of the conductance activated by prehyperpolarization to −100 mV. $K^+$-free high-$Cl^-$ $Cs^+$ internal solution; $K^+$-free external solutions. (*A*) Voltage jump protocol used to record this tail current at −40, −20, 0, +20 and +40 mV. (*B*) Current traces recorded during hyperpolarizing jumps from 0 to −100 mV after 20–30 min of whole-cell recording, during the period of stability of the slow inward relaxation, in the Na-0 K (Cl) external solution, and after substitution of most external $Cl^-$ ions by an impermeant anion cyclamate, in the Na cyclamate-0 K external solution. The amplitude of the relaxation was little affected, whereas its kinetics of activation was slowed by the $Cl^-$-cyclamate substitution. (*C* and *D*) Tail currents recorded during repolarization (between −40 and +40 mV) after the prepulse to −100 mV, in the Na-0 K solution (*C*) and in the Na cyclamate-0 K solution (*D*). The slow tail current reverses close to 0 mV ($E_{Cl}$) in *C* and remains inward up to +40 mV in *D*, where $E_{Cl}$ was +80 mV.

lowed by repolarization to a variable potential) and $K^+$-free internal and external solutions (high-$Cl^-$ $Cs^+$ internal solution, Na-0 K or Na cyclamate-0 K external solution). The tail current corresponding to the deactivation of the hyperpolarization-activated current was thus recorded between −40 and +40 mV, both when $E_{Cl}$ was −1 mV (Na-0 K external solution; Fig. 2*C*) and when $E_{Cl}$ was +80 mV (Na cyclamate − 0 K external solution; Fig. 2*D*). The inward relaxations recorded at −100 mV in the two external solutions are shown in Fig. 2*B*. In the Na-0 K solution, repolarization from −100 mV induced a slow tail current reversing around 0 mV (Fig. 2*C*). That this slow tail current corresponded to the deactivation of the current slowly activated by the prepulse to −100 mV was shown by the evolution with time of its amplitude, which followed that of the relaxation recorded at −100 mV (in particular, no such slow tail current was observed when hyperpolarizing jumps did not induce a slow inward relaxation; *not shown*). The additional transient inward current observed during the first 100 msec at −40, −20 and 0 mV could be completely blocked by substitution of the 2 mM external $CaCl_2$ by $MgCl_2$ (three experiments, *not shown*), and thus is the transient calcium current previously described [10]. In the Na cyclamate-0 K solution, a slow tail current was still observed but it no longer reversed over the voltage-range investigated (Fig. 2*D*). (Above +40 mV, the membrane usually deteriorated, as indicated by large erratic conductance changes).

The reversal potential change from 0 mV in the Na-0 K solution to a value more depolarized than +40 mV

in the Na cyclamate-0 K solution clearly shows that the current investigated is carried by $Cl^-$ ions.

### Voltage-dependent Activation of the Current

A voltage-jump protocol designed to establish activation *I–V* curves was applied in the Na-0 K external solution, using the $K^+$-free high-$Cl^-$ $Cs^+$ internal solution. A series of hyperpolarizing jumps from 0 mV (to −20, −30, −40, −60, −80, −100 mV, and in some cases down to −160 mV) was applied regularly to observe the evolution with time of the relaxation. The relaxation induced by a given hyperpolarization increased steeply in amplitude during the first minutes of the recording and then leveled off, being usually quite stable for several tens of minutes. Its disappearance, if it occurred, was observed much later than with the $K^+$-containing solutions and the regular jumps to −100 mV. The long period of stability of the hyperpolarization-activated current under the conditions used to establish *I–V* curves allowed us to perform the other experiments described below (pharmacological study and study of the osmosensitivity).

Fig. 3*A* shows the successive current traces recorded during hyperpolarizing jumps from 0 mV to various membrane potentials during the period of stability of the relaxation. Each hyperpolarizing jump induced a time-dependent increase in inward current. The amplitude of this relaxation increased steeply with the degree of hyperpolarization. Furthermore, the time course of the relaxation induced by each hyperpolarization became more rapid for stronger hyperpolarizations. In an attempt to quantify these properties, we fitted each relaxation with one or two exponential components (*see* Materials and Methods). Good monoexponential fits were obtained at −20, −30 and −40 mV, whereas only biexponential fits were satisfactory at −60 mV and below. The time constants and the relative amplitude of the two components are given by Table 2. In each experiment, the total current recorded after 10 sec hyperpolarization, and the amplitude of the hyperpolarization-activated relaxation (estimated by exponential fit: rapid phase ($A_f$) + slow phase ($A_s$)) were both measured over the whole voltage range (*see* Fig. 3B). Both the total current and the relaxation amplitude showed a pronounced inward rectification, reflecting the voltage dependence of the underlying hyperpolarization-activated $Cl^-$ conductance. By dividing the relaxation amplitude ($A_f + A_s$) by the electrochemical driving force for $Cl^-$ ions ($V - E_{Cl}$), one can obtain estimates of this conductance $g(V)$ (or more precisely, of the increase in conductance between the holding potential 0 mV and the test potential $V$, $\Delta g(V) = g(V) - g(O)$). Its absolute value was variable from one cell to the other, but its voltage-dependence was reproducible. For the 12 similar experiments performed in $K^+$-free solutions, the ratio of the relaxation amplitudes was 11.9 ± 2 between −60 and −20 mV and 5.2 ± 1.1 between −60 and −30 mV). This means that the conductance increased by at least a factor of 4 between −20 and −60 mV and by a factor of 2.6 between −30 and −60 mV. The amplitude of the relaxation recorded at −60 mV varied between 88 and 393 pA (mean 215 ± 115 pA, which leads to $\Delta g(-60)$ = 3.6 ± 1.9 nS). The first ten milliseconds of the $Cl^-$ relaxation are obscured by imperfectly compensated capacitive currents. This did not prevent estimation of the relaxation amplitude for moderate hyperpolarizations. However, that the kinetics of activation of the $Cl^-$ relaxation becomes faster with increasing hyperpolarization (*see* Table 2) makes the measurements more

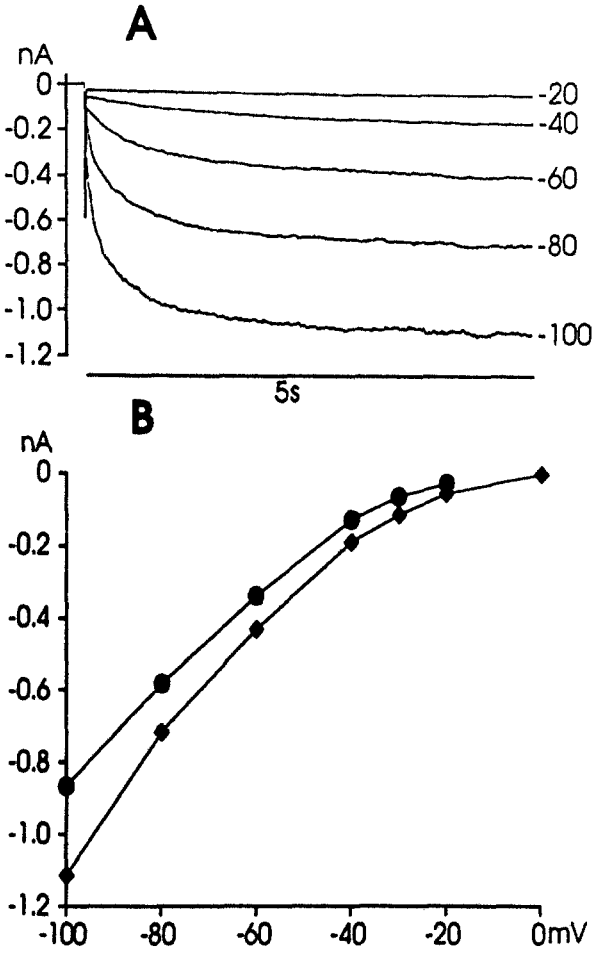


**Fig. 3.** *I–V* curve of the $Cl^-$ current activated by hyperpolarization. High $Cl^-$ $Cs^+$ internal solution; Na-0 K external solution. (*A*) Current traces recorded during a series of hyperpolarizing jumps from 0 mV to the potentials indicated on the right. (*B*) Corresponding *I–V* curves measured either at the end of the 10 sec hyperpolarizing jumps (◆), or by measuring the amplitude of the relaxation activated by each hyperpolarization from its exponential fit (●).

**Table 2.** Voltage sensitivity of the kinetics of activation of the hyperpolarization-activated $Cl^-$ current.

| Experimental condition | $V$ (mV) −20 | −30 | −40 | −60 | −80 | −100 |
|---|---|---|---|---|---|---|
| $\tau_s$ (sec) | 3.31 ± 0.53 | 2.82 ± 0.46 | 2.19 ± 0.35 | 1.93 ± 0.44 | 1.62 ± 0.37 | 1.47 ± 0.40 |
| $\tau_f$ (msec) | | | | 270 ± 60 | 206 ± 32 | 160 ± 40 |
| $A_f/(A_f + A_s)$ | | | | 0.44 ± 0.11 | 0.58 ± 0.05 | 0.62 ± 0.06 |

The current relaxations recorded in 12 similar experiments during a series of hyperpolarizing jumps from 0 mV (in the Na-0 K external solution, high-$Cl^-$ $Cs^+$ internal solution, during the period of stability of the relaxation) have been fitted with exponentials. Good monoexponential fits were obtained for jumps to −20, −30, and −40 mV, whereas traces recorded during stronger hyperpolarizations could be fitted in a satisfactory way only by the sum of two exponential components. The time constants, ($\tau_s$ and $\tau_f$), as well as the relative amplitude of the fast component ($A_f/(A_f + A_s)$) derived from these fits are given as a function of the potential $V$ at which the relaxation was recorded. Both time constants became smaller with stronger hyperpolarizations; furthermore the relative amplitude of the fast component increased markedly, from being undetectable above −40 mV to more than 50% below −80 mV.

difficult below −100 mV. Furthermore, the amplitude of the $Cl^-$ relaxations induced by strong hyperpolarizing jumps usually becomes large enough to introduce a significant voltage clamp error due to the series resistance. These limitations explain why we do not show $I$–$V$ curves below −100 mV.

### Pharmacological Properties

External addition of niflumic acid at 20 μM (two experiments performed with the $K^+$-containing solutions) did not affect the $Cl^-$ relaxation activated by hyperpolarization from 0 to −100 mV. However, this compound induced an outward current at 0 mV ($E_{Cl}$), an effect likely to result from a $K^+$ conductance increase (*see ref.* 43).

The effects of external diphenyl carboxylate, DPC at 1 mM, were investigated in four cells using $K^+$-containing solutions (Na-5 K external solution, high-$Cl^-$ $K^+$ internal solution). In one of these cells, DPC induced a large conductance increase (*not shown*) preventing a reliable estimation of its effect on the $Cl^-$ relaxation. In the three remaining cells, DPC did not affect the $Cl^-$ relaxation. This was confirmed by two additional experiments performed in $K^+$-free solutions (Na-0 K external solution, high-$Cl^-$ $Cs^+$ internal solution).

The effects of external DIDS were first investigated with $K^+$-containing solutions. DIDS (1 mM) reduced the $Cl^-$ relaxation recorded at −100 mV, but it also activated an outward current at 0 mV likely to result from a $K^+$ conductance increase (*see ref.* 31). Thus, the effect of DIDS on the $Cl^-$ relaxation was then studied in $K^+$-free solutions.

Figure 4*A* shows the current traces recorded during hyperpolarizing jumps from 0 to −40 or −100 mV in control and in the presence of DIDS 1 mM. The $Cl^-$ relaxation was markedly slowed and reduced in amplitude at −100 mV. It was completely blocked at −40 mV. That the blocking effect of DIDS was more pronounced at a less negative membrane potential was observed in three similar experiments. The results are summarized by Fig. 4*B* which shows, as a function of membrane potential, the DIDS-induced reduction of the $Cl^-$ relaxation amplitude. The effect of DIDS on the $Cl^-$ relaxation was not completely reversible on a time scale of a few minutes (*not shown*). As shown above, external DIDS, even at 1 mM, was not able to completely suppress the $Cl^-$ relaxation over the whole voltage range. In some other preparations, application of DIDS to the intracellular face of the membrane has been shown to be much more effective than external application of DIDS (*see ref.* 37). In six experiments we used an internal solution (high-$Cl^-$ $K^+$) supplemented with DIDS (50 μM in two cells, 100 μM in three cells, 1 mM in one cell). This did not prevent the usual development of the $Cl^-$ relaxation. (However, slight partial inhibitory effects of internal DIDS cannot be excluded).

Another standard $Cl^-$ channel blocker, NPPB, was found to reduce the $Cl^-$ current activated by hyperpolarization. The blocking effect of 100 μM NPPB on the current activated by a jump from 0 to −100 mV is illustrated by Fig. 4*C*. In three similar experiments, the amplitude of the relaxation recorded at −100 mV was reduced by 31 ± 2%. This effect of NPPB was rapidly reversible (*see wash* in Fig. 4*C*) and was not markedly voltage dependent (*not shown*).

$Cd^{2+}$ ions were found to be very potent blockers of the $Cl^-$ current studied. The blocks induced by 20 and 300 μM $Cd^{2+}$ are illustrated in Fig. 5*A* and *B*. These effects were completely reversible after washout of $Cd^{2+}$ ions (Fig. 5*C*). $Cd^{2+}$ reduced the amplitude of the $Cl^-$ relaxation recorded at −100 mV in $K^+$-free solutions (Na-0 K external solution, high-$Cl^-$ $Cs^+$ internal solution) by 37% ± 7% (6) at 20 μM and by 74% ± 16% (3) at 100 μM. Complete block was induced at 1 mM (six experiments with the Na-5 K external solution and

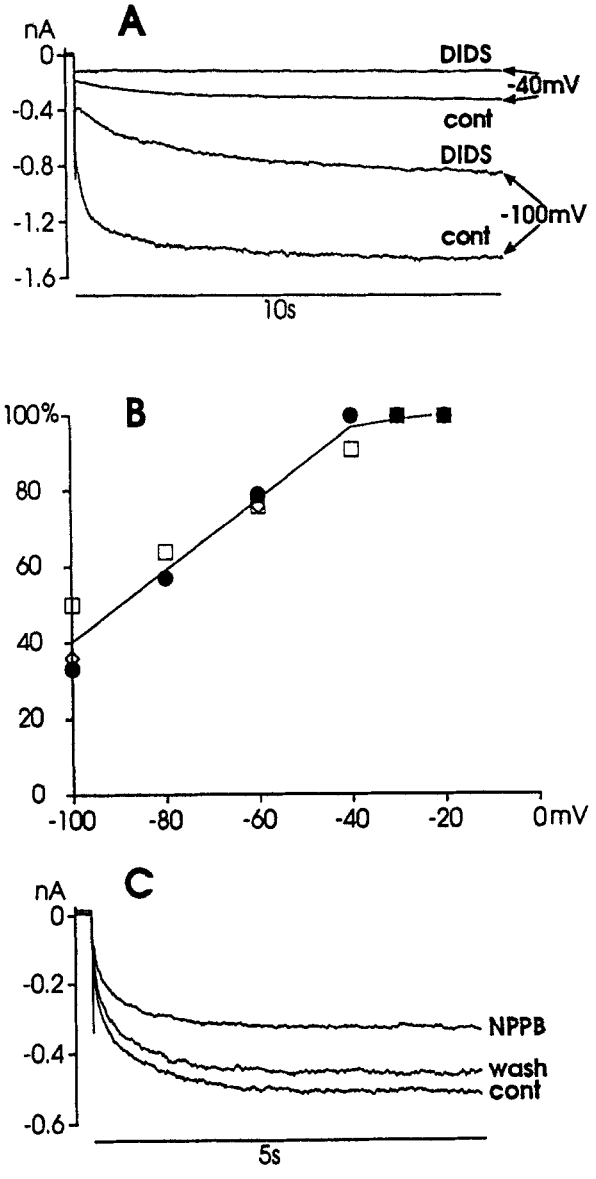


**Fig. 4.** Blocking effect of DIDS (*A* and *B*) and NPPB (*C*) on the hyperpolarization-activated $Cl^-$ current. Na-0 K external solution, high-$Cl^-$ $Cs^+$ internal solution. (*A*) Current traces recorded during hyperpolarizing jumps from 0 to −40 or −100 mV, before or 6–7 min after the addition of 1 mM DIDS to the bath. (*B*) Percent reduction of the amplitude of the relaxation recorded at various membrane potentials, *V*, by 4–6 min 1 mM external DIDS, as a function of *V*. Different symbols represent three different experiments and the curve has been drawn through the mean values. (*C*) Current traces recorded during jumps from 0 to −100 mV before (*cont*), during (*NPPB*) and after (*wash*) an application of 100 μM NPPB. The $Cl^-$ relaxation was reduced by NPPB but was not completely blocked, even after more than 10 min in its presence.

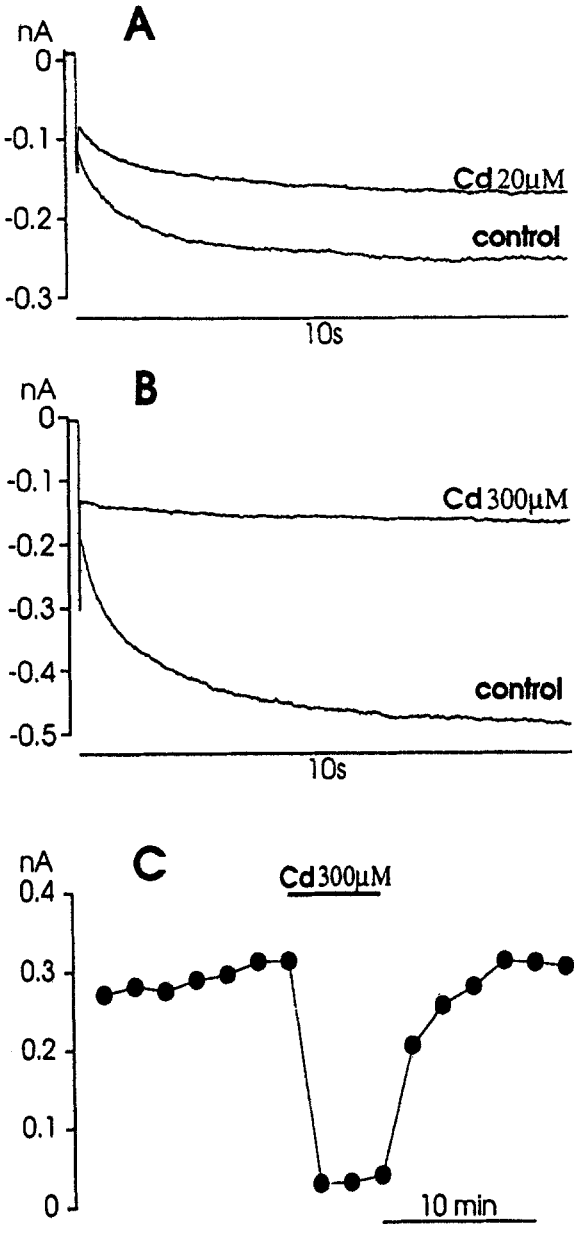


**Fig. 5.** Reversible block of the hyperpolarization-activated $Cl^-$ current by low concentrations of extracellular cadmium ions. Na-0 K external solution, high-$Cl^-$ $Cs^+$ internal solution. (*A* and *B*) Current traces recorded in two different cells during voltage jumps from 0 to −60 mV, *A*, in the absence and presence of $Cd^{2+}$, 20 μM (*A*) or 300 μM (B). (*C*) Plot of the relaxation amplitude during successive jumps to −60 mV in the experiment illustrated in *B*. First point obtained 90 min after beginning of whole-cell recording.

the high-$Cl^-$ $K^+$ internal solution, one experiment with the $K^+$-free solutions). The blocking effect of $Cd^{2+}$ was similar over the whole voltage range.

This blocking effect was not a consequence of the block of calcium currents since the $Cl^-$ relaxation was completely insensitive to the removal of external $Ca^{2+}$ ions (substitution by $Mg^{2+}$ ions, three experiments). This $Cl^-$ current was also insensitive to $Ba^{2+}$ ions (1 mM, four experiments) or $Sr^{2+}$ ions (300 μM, one experiment).

## The $Cl^-$ Current Activated by Hyperpolarization is Distinct from a $Cl^-$ Current Activated by Extracellular Hyposmolarity

The experiments described below were all performed in $K^+$-free solutions to avoid contamination of the records by possible osmosensitive $K^+$ currents (*see ref.* 16 for a description of osmosensitive $K^+$ channels in an osteoblastic cell line). The internal solution was the usual high-$Cl^-$ $Cs^+$ solution. To be able to reduce the external osmolarity without changing the ionic concentrations, we used a $K^+$-free sucrose-containing external solution, the 105 Na-70 sucrose solution.

In osteoblasts, reducing the osmolarity of the extracellular solution induced a conductance increase very similar to the $Cl^-$ conductance activated by external hyposmolarity in other cell types (*see e.g., ref.* 17).

This conductance is illustrated in Fig. 6*A* by current traces recorded in the 105 Na-70 sucrose and 105 Na-0 sucrose solutions during voltage jumps of small amplitude (from 0 to −20 mV, to avoid the activation of large hyperpolarization-induced currents) and in Fig. 6*B* by the corresponding "instantaneous" *I–V* curves. These curves were obtained by measuring the current as soon as possible after the onset of each hyperpolarizing jump, thus minimizing the contribution of the slow relaxation activated by hyperpolarization. (The effects of sucrose removal on the hyperpolarization-activated current are described in the next section by Fig. 7).

The removal of 70 mM external sucrose induced a similar conductance increase in six other experiments. The instantaneous inward current measured at −40 mV was increased by 870 ± 280 pA by sucrose removal. In two additional experiments, we observed that the removal of only 20 or 35 mM sucrose also induced a conductance increase. In some of these experiments, depolarizing jumps from 0 to +20, +30 and/or +40 mV were also applied (in addition to the usual hyperpolarizing jumps) and revealed that the hyposmolarity-induced current reversed close to the $Cl^-$ equilibrium potential (7 mV with the 105 Na-70 or 0 sucrose external solutions and the high-$Cl^-$ $Cs^+$ internal solution) and showed some outward rectification (*not shown*).

In four cells, we used the low-$Cl^-$ $Cs^+$ internal solution, instead of the high-$Cl^-$ $Cs^+$ internal solution. As expected from the results obtained with the low-$Cl^-$ $K^+$ internal solution, none of these cells showed the $Cl^-$ current activated by hyperpolarization. In contrast, the current activated by the removal of 70 mM external sucrose persisted under these ionic conditions (Figs. 6*C* and *D*). Its reversal potential was shifted towards negative membrane potentials by the substitution of internal $Cl^-$ by glutamate ions, as expected for a current mainly carried by $Cl^-$ ions.

The outwardly rectifying $Cl^-$ current, activated by hypotonic external solutions in HeLa cells [17] or in fibroblasts permanently transfected with the multidrug-resistance P-glycoprotein [53], is sensitive to a forskolin analogue, DDFSK, which does not interact with adenylate cyclase. Similarly, the current activated by external hyposmolarity in osteoblastic cells was completely blocked by 80 μM DDFSK. This result, confirmed on four different cells, is illustrated by the trace and curve *3* of Fig. 6*A* and *B*, obtained in the 105 Na-0 sucrose solution in the presence of DDFSK. They are almost superimposed with the control traces (*1*) observed before sucrose removal. This blocking effect of DDFSK was partly reversible, as shown by the results labeled *4*, obtained after wash of DDFSK while the cell was still bathed in the 0 sucrose solution. In an additional experiment where DDFSK was applied at only 16 μM, it reduced by about a half the current activated by hyposmolarity (*not shown*) and this effect was completely reversible upon washout of DDFSK.

The hyposmolarity-induced current was also strongly reduced (by more than 80%) by the addition of 1 mM DIDS to the 0 sucrose external solution (*not shown*).

The sensitivity to DDFSK of the current activated by hyposmolarity allowed us to clearly separate pharmacologically this current from the $Cl^-$ current activated by hyperpolarization. Indeed, over the whole voltage range, the latter current was insensitive to 80 μM DDFSK (seven experiments, performed either in the 105 Na-70 sucrose external solution or in the Na-0 K solution). This is illustrated by Fig. 6*E* and *F*.

## The $Cl^-$ Current Activated by Hyperpolarization Is Reduced by External Hyposmolarity and Is Increased by External Hyperosmolarity

Reducing the external osmolarity by removal of 70 mM external sucrose not only induced a DDFSK-sensitive conductance increase but also markedly reduced the slow $Cl^-$ relaxation activated by hyperpolarization. This observation is illustrated by Fig. 7*A* showing current traces recorded during hyperpolarizing jumps from 0 to −100 mV.

This result, obtained in all the experiments performed, could be difficult to interpret because of the superposition of two distinct currents. Thus, we further investigated the effects of reducing the external osmolarity under conditions preventing the development of the usual hyposmolarity-induced conductance increase.

In two experiments, 80 μM DDFSK was added both in the 105 Na-70 sucrose solution and in the 105 Na-0 sucrose solution. Under these conditions, sucrose removal did not induce any conductance increase, but markedly reduced the $Cl^-$ relaxation induced by hyperpolarizing jumps (Fig. 7*B*). The reduction of the re-

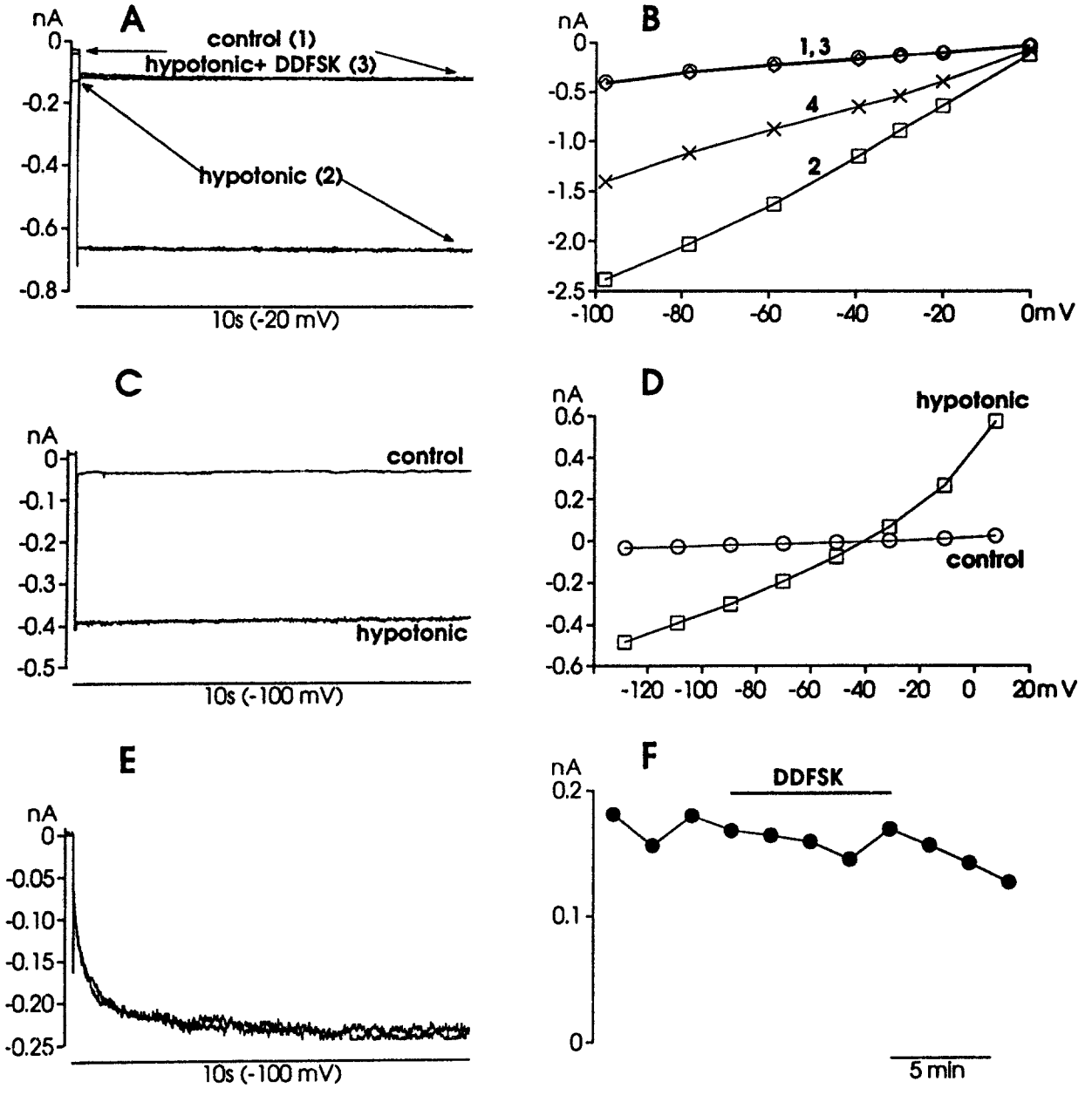


**Fig. 6.** The hyperpolarization-activated $Cl^-$ current is different from the current activated by reducing the external osmolarity. The external solution was either the 105 Na-70 sucrose solution (traces *1* in *A* and *B*; *control* traces) or the 105 Na-0 sucrose solution (traces *2* and *4* in *A* and *B*, *hypotonic* in *C* and *D*). (*A* and *B*) (same cell; high-$Cl^-$ $Cs^+$ internal solution). Conductance increase, induced by reducing the external osmolarity (traces *1* and 2) and its reversible blockade by addition of DDFSK (80 μM) to the hypotonic solution (*3:* 105 Na-0 sucrose + DDFSK; *4:* after 4–6 min wash with the 105 Na-0 sucrose solution in the absence of DDFSK). (*A*) Current traces recorded during voltage jumps from 0 to −20 mV. (*B*) Corresponding "instantaneous" *I–V* curves, obtained by measuring the current at 0 mV and 30 msec after the onset of each hyperpolarization from 0 mV to the potential *V*. (*C* and *D*) Conductance increase induced by reducing the external osmolarity in a cell dialysed with the low-$Cl^-$ $Cs^+$ internal solution ($E_{Cl}$ = −52 mV). (*C*) Current traces recorded during voltage jumps to −110 mV (control trace obtained 70 min after beginning of whole-cell recording). (*D*) Corresponding instantaneous *I–V* curve (current measured 100 msec after the onset of each voltage jump). (*E* and *F*) Addition of DDFSK (80 μM) to the 105 Na-70 sucrose external solution (high-$Cl^-$ $Cs^+$ internal solution) does not affect the hyperpolarization-activated $Cl^-$ current. (*E*) Current traces recorded during voltage jumps from 0 to −100 mV. (*F*) Corresponding plot of the amplitude of the $Cl^-$ relaxation recorded during successive jumps to −100 mV (first point obtained 87 min after beginning of whole-cell recording).

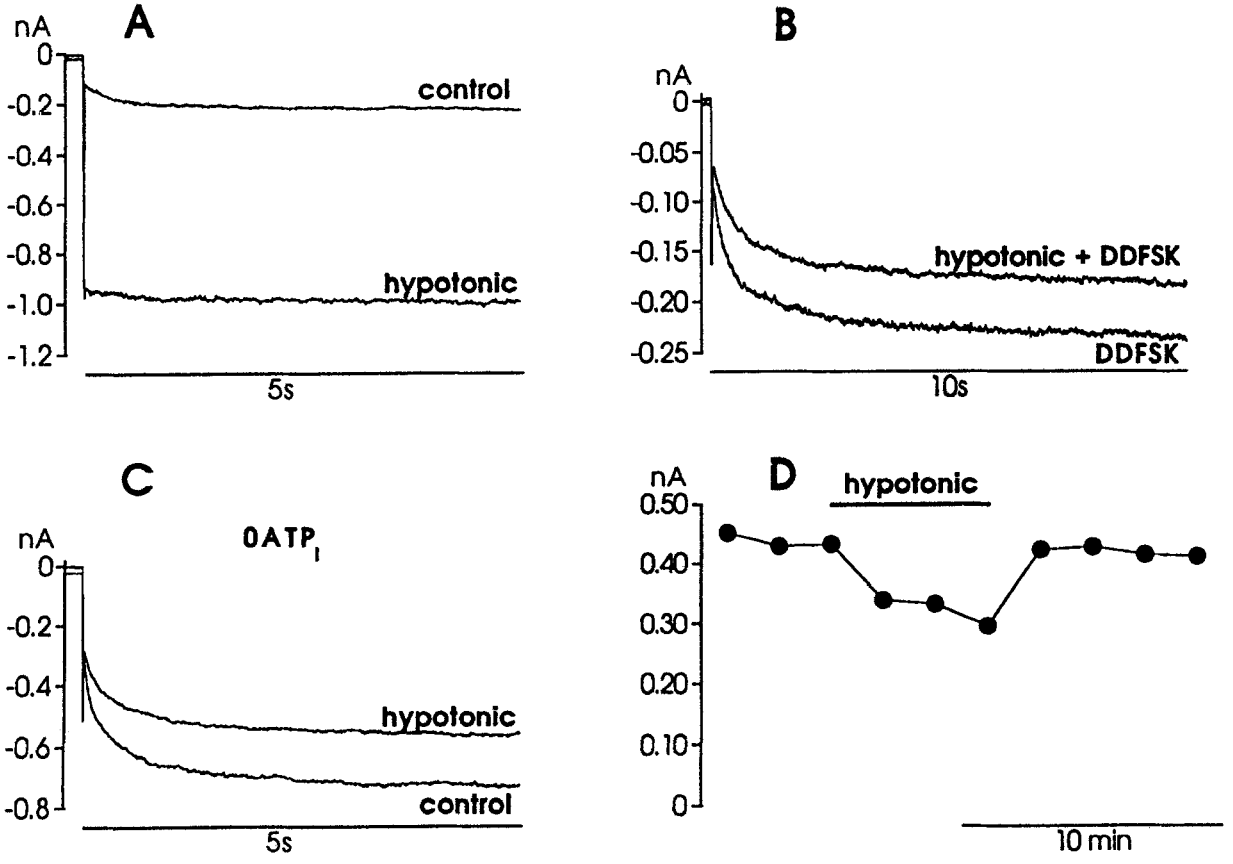


**Fig. 7.** The $Cl^-$ current activated by hyperpolarization is reversibly reduced by lowering the external osmolarity. High-$Cl^-$ $Cs^+$ internal solution; 105 Na-70 sucrose or 105 Na-0 sucrose external solution. (*A*) Experiment performed in the absence of DDFSK. Current traces recorded during voltage jumps from 0 to −100 mV, in the presence (*control*) and absence (*hypotonic*) of 70 mM external sucrose. (*B*) Experiment performed in the presence of 80 μM DDFSK in both external solutions. Current traces recorded during voltage jumps from 0 to −60 mV in the presence of both sucrose and DDFSK (*DDFSK*) and after sucrose removal (*hypotonic + DDFSK*). (*C* and *D*) Experiment performed with a pipette solution containing no ATP. (*C*) Current traces recorded during voltage jumps from 0 to −80 mV before and after sucrose removal. (*D*) Plot of the relaxation amplitude recorded during successive jumps from 0 to −80 mV. First point obtained 67 min after the beginning of whole-cell recording. Note that both in the presence of DDFSK (*B*) and in the absence of internal ATP (*C*), sucrose removal even reduced the total inward current recorded at the end of the hyperpolarizing jumps. The slight current change induced at 0 mV by sucrose removal in *B* and *C* is outwardly directed, in agreement with the idea that already at 0 mV, a small inward $Cl^-$ current could be activated ($E_{Cl}$ being close to +7 mV) and reduced by sucrose removal.

laxation amplitude was more pronounced for less negative membrane potentials (*see* Table 3). In another experiment, in which no ATP was added in the internal solution, the effects of removal and later restoration of sucrose were tested three times in succession. Whereas a reversible instantaneous conductance increase was induced by the first reduction of external osmolarity, this effect was smaller during the second test and it no longer occurred during the third. This result (which is in agreement with the ATP dependence of the activation of a $Cl^-$ current by external hypotonicity in other cells [17]) allowed us to separate in another way the effect of external hyposmolarity on the slow $Cl^-$ relaxation from its usual effect on the instantaneous conductance. The amplitude of the $Cl^-$ relaxation was reduced by the third hypotonicity challenge, whereas the instantaneous conductance was very little affected (Fig. 7*C*). The effect of sucrose removal on the hyperpolarization-activated current was always reversible during wash with the sucrose-containing solution (Fig. 7*D*).

The first line of Table 3 summarizes the effect of sucrose removal on the amplitude of the $Cl^-$ relaxation in the three experiments where it did not induce an instantaneous conductance increase.

The kinetics of activation of the hyperpolarization-activated current was slower after sucrose removal: e.g., the time constant derived by monoexponential fit of the $Cl^-$ relaxation recorded at −40 mV was increased by 32 ± 3% in the three experiments of Table 3.

In eight experiments, we investigated the sensitivity of the hyperpolarization-activated $Cl^-$ current to an *increase* of the external osmolarity. This was done by

**Table 3.** Voltage sensitivity of the effects of changes in the external osmolarity on the amplitude of the $Cl^-$ relaxations activated by a series of hyperpolarizing jumps from 0 mV.

| Experimental condition | $V$ (mV) | | | | | |
|---|---|---|---|---|---|---|
| | −20 | −30 | −40 | −60 | −80 | −100 |
| %↘ (iso → hypo) | 54 ± 2 | 46 ± 3 | 37 ± 0.8 | 35 ± 2 | 25 ± 2 | 22 ± 3 |
| %↗(iso → hyper) | 74 ± 26 | 35 ± 3 | 31 ± 8 | 21 ± 4 | 23 ± 7 | 11 ± 5 |

First line: percentage decrease of the relaxation induced by reducing the external osmolarity by removal of the 70 mM sucrose from 105 Na-70 sucrose external solution (mean of three experiments where no instantaneous conductance increase was induced, either because of the continuous presence of 80 μM DDFSK, or because of the absence of internal ATP). Second line: percentage increase of the relaxation induced by increasing the external osmolarity by addition of 42 mM sucrose to the Na-0 K external solution (iso → hyper: mean of three experiments). The amplitude of each relaxation was measured from its exponential fit.

addition of 42 mM sucrose to the Na-0 K solution, using the high-$Cl^-$ $Cs^+$ internal solution. Increasing the external osmolarity increased the current and this effect was reversible (Fig. 8). The change of the $Cl^-$ current activated by hyperpolarization was not correlated with any pronounced change of the instantaneous conductance. As reported above for a decrease of the external osmolarity, the sensitivity of the $Cl^-$ relaxation to an increase of the external osmolarity was more pronounced at less negative membrane potentials. The average results of three experiments where the $Cl^-$ relaxation could be precisely measured over a wide voltage range, both in the usual Na-0 K external solution and in the Na + sucrose external solution, are given on the second line of Table 3. The kinetics of activation of the hyperpolarization-activated current was faster after addition of sucrose: the time constant derived from monoexponential fits of the relaxations recorded at −40 mV was decreased by 18 ± 6% (3).

Various mechanosensitive ionic channels are known to be sensitive to gadolinium ions ($Gd^{3+}$) [28, 56]. The osteoblastic hyperpolarization-activated $Cl^-$ current was not affected by the external addition of 10–100 μM $Gd^{3+}$ (two cells). Furthermore, the presence of 100 μM external $Gd^{3+}$ did not prevent its increase by addition of sucrose to the external solution.

## Discussion

The present study has revealed that in addition to their various voltage-gated currents activated by depolarization (calcium currents [1, 10, 22, 26, 27, 39], sodium currents [10], and potassium currents [12, 16, 44, 58]), osteoblastic cells also have a current slowly activated by hyperpolarization. This current is distinct from the nonselective cationic current slowly activated by hyperpolarization in some other cell types (called $I_f$, $I_Q$ or $I_h$ according to the authors, *see refs.* 19 and 36 and included references), since it is not affected by external $Na^+$ removal, persists in $K^+$-free solutions, and is not blocked by external $Cs^+$ ions. It is carried by $Cl^-$ ions, as shown by tail currents recorded when $E_{Cl}$ was 0 or +80 mV (Fig. 2), and by its very strong sensitivity to the intracellular $Cl^-$ concentration. Whereas slow relaxations of several hundreds of pA could be reproducibly recorded at −100 mV when $E_{Cl}$ was close to 0 mV in high-$Cl^-$ solutions (142 mM internal $Cl^-$), no relaxation at all was recorded when $E_{Cl}$ was between −50 and −60 mV after replacement of internal $Cl^-$ by glutamate (13–14 mM internal $Cl^-$). This difference suggests that, as previously demonstrated in the case of the channel of *Aplysia* neurons [8], the intracellular anions may modulate the gating properties of the hyperpolarization-activated channel. This remains to be supported by further studies allowing comparison of the threshold of activation of the current for various intracellular $Cl^-$ concentrations.

The $Cl^-$ conductance responsible for the hyperpolarization-activated current is steeply voltage dependent. The amplitude of the relaxation increased by a factor close to 12 between −20 and −60 mV, that is for only a three-fold increase in driving force. Its kinetics of activation is also markedly voltage dependent. It becomes more rapid for stronger hyperpolarizations.

In a previous study performed on the same cells [11], we described an outwardly rectifying $Cl^-$ current activated by the adenylate cyclase pathway, detectable over the whole voltage range. Most of the experiments of this previous study were performed using low-$Cl^-$ internal solutions and very short voltage jumps, so they would not have revealed the slow $Cl^-$ relaxation studied here.

### Pharmacological Properties

We have shown that the hyperpolarization-activated $Cl^-$ current is insensitive to 1 mM external DPC and can be blocked by 1 mM external DIDS in a voltage-dependent manner, the blocking effect of DIDS being more pronounced at less negative membrane potentials.

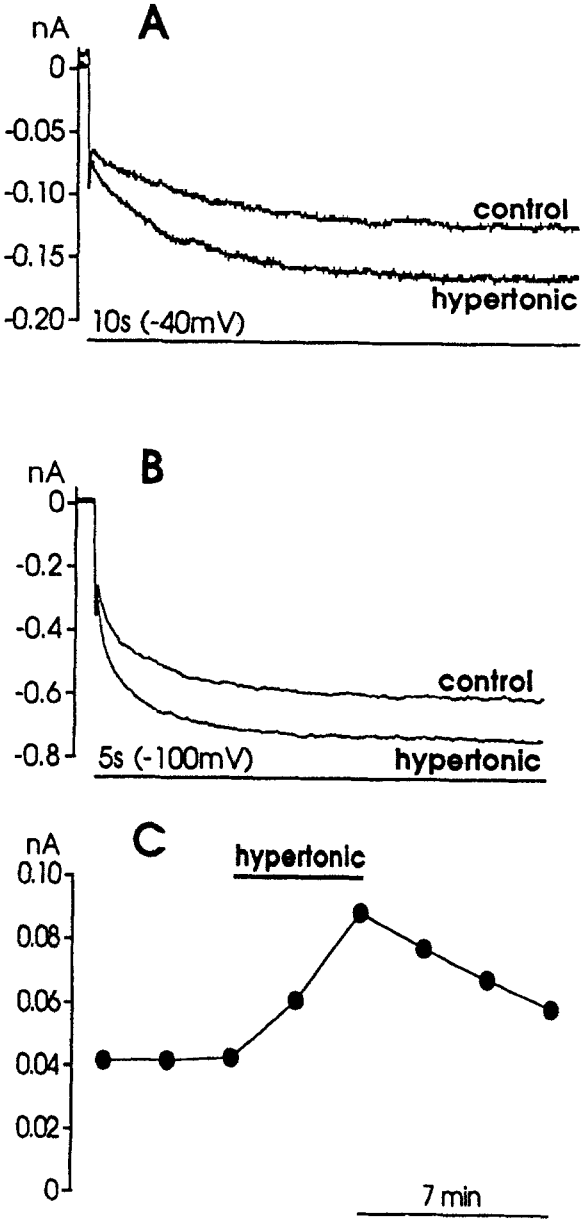


**Fig. 8.** The hyperpolarization-activated $Cl^-$ current is reversibly increased by increasing the external osmolarity. Experiment performed with the high-$Cl^-$ $Cs^+$ internal solution using the *control* Na-0 K solution or a *hypertonic* solution, the Na + sucrose solution. (*A* and *B*) Current traces recorded during voltage jumps from 0 to −40 mV (*A*) or −100 mV (*B*) in both solutions. Note that in the hypertonic external solution, the total inward current recorded at the end of each hyperpolarizing jump was larger than in control, showing that external hyperosmolarity increased the total conductance. (*C*) Plot of the relaxation amplitude measured for each successive jump from 0 to −40 mV.

These properties differ from the pharmacological properties reported for two hyperpolarization-activated $Cl^-$ channels recently cloned, ClC0 (DPC sensitive [30]) and ClC2 (both DPC sensitive and almost DIDS insensitive [51]).

The osteoblastic hyperpolarization-activated $Cl^-$ current is very sensitive to external $Cd^{2+}$ ions, as the hyperpolarization-activated $Cl^-$ conductance of some rat neurons [34, 48]. The $Cd^{2+}$-sensitivity of the hyperpolarization-activated $Cl^-$ current is not secondary to calcium current blockade, since this $Cl^-$ current is insensitive to the removal of external $Ca^{2+}$ ions. In hippocampal rat neurons, protein kinase C stimulation by phorbol esters has been shown to block the hyperpolarization-activated $Cl^-$ current [34]. Thus, it is possible that the strong $Cd^{2+}$-sensitivity of the in vitro activity of various types of protein kinases C [47] may be responsible for the surprising $Cd^{2+}$-sensitivity of this type of $Cl^-$ current.

The insensitivity of the osteoblastic hyperpolarization-activated $Cl^-$ current to 1 mM $Ba^{2+}$ and its strong $Cd^{2+}$-sensitivity differ from the $Ba^{2+}$-sensitivity (50% block by 0.36 mM $Ba^{2+}$) and low $Cd^{2+}$-sensitivity (17% block by 1 mM $Cd^{2+}$) of the hyperpolarization-activated $Cl^-$ current recorded in *Xenopus* oocytes after expression of phospholemman [38].

### Osmosensitivity of the Hyperpolarization-activated $Cl^-$ Current

Several previous studies, performed with the whole-cell recording technique on other cell types, have shown that reducing the external osmolarity activates a $Cl^-$ current which either does not show any voltage dependence or shows some inactivation during very strong depolarizing jumps [5, 17, 21, 32, 33, 35, 49, 53, 54].

As expected from these data, we observed that reducing the external osmolarity activates a large current, whose reversal potential was close to $E_{Cl}$ when using the high-$Cl^-$ $Cs^+$ internal solution and was shifted in the same direction as $E_{Cl}$ after substitution of internal $Cl^-$ ions by glutamate ions. (The slight difference between this reversal potential and $E_{Cl}$ when using the low-$Cl^-$ $Cs^+$ internal solution (Fig. 6*D*) could result from a small permeability of the channels to glutamate ions).

The complete blockade of the osteoblast hypotonicity-activated current by 80 μM DDFSK and the total insensitivity of the hyperpolarization-activated $Cl^-$ current to this agent clearly show that these two currents are pharmacologically distinct. Their sensitivity to the intracellular $Cl^-$ concentration was also strikingly different, since reducing the internal $Cl^-$ concentration (by substitution with glutamate ions) did not prevent the development of the hypotonicity-induced current but totally abolished the hyperpolarization-activated current. In agreement with previous results on other cell types [17, 33], we also observed that prolonged intracellular dialysis with a pipette solution without added ATP could eliminate the hypotonicity-activated current, whereas the hyperpolarization-activated current

persisted. Note that a recent study of the effects of external hypotonicity on osteoblast-like cells (G292), which was performed both in $K^+$-containing solutions and in the absence of internal ATP, showed that external hypotonicity activates a $K^+$ current (that we could not detect using $K^+$-free solutions), but did not mention the activation of a $Cl^-$ current, which could be explained by the absence of internal ATP [16].

After elimination of the current activated by external hypotonicity (using either DDFSK or an ATP-free internal solution), we found that reducing the external osmolarity both reduced the amplitude of the hyperpolarization-activated $Cl^-$ relaxation and slowed its kinetics of activation. This osmosensitivity was confirmed by the results of experiments showing that, in contrast, increasing the external osmolarity both increased the amplitude of the $Cl^-$ relaxation and accelerated its kinetics of activation.

The modulations of the amplitude of the $Cl^-$ relaxation induced by reducing or increasing the external osmolarity were both voltage dependent (more pronounced for smaller hyperpolarizations). These results cannot be explained only by an osmosensitivity of the total number of active channels, but show that the voltage dependence of activation of the channels is sensitive to osmolarity. The present analysis, however, is limited by the difficulty of measuring the relaxation amplitude in a reliable way over a voltage range large enough to reach clear saturation of the conductance.

The osmosensitivity of the hyperpolarization-activated $Cl^-$ current studied in the present paper contrasts with that reported for ClC2, and ClC2 remains the only example of a channel activated both by external hypotonicity and by hyperpolarization from a holding potential $\leq 0$ mV [25]. However, that it is not the same channel which is activated by external hypotonicity and by hyperpolarization from 0 mV in osteoblastic cells is in agreement with the known voltage dependence of hypotonicity-activated $Cl^-$ currents in other cell types (*see e.g., ref.* 32 pointing out that the apparent time-dependent activation below $-60$ mV of a hypotonicity-induced $Cl^-$ current in fact represented the release from the inactivation induced by previous large positive pulses).

Two reports have recently described conductances activated by hypertonic external solutions. One of them, discovered in airway epithelial cells, is a chloride-dependent cation conductance which can be completely blocked by the addition of 10 μM gadolinium to the external solution [6]. The second one has been observed in vasopressin-releasing hypothalamic neurons and is responsible for the depolarization and consequent accelerated action potential discharge induced by external hypertonicity in these osmoreceptor neurons [40]. The channels responsible for this conductance have also been reported to be nonselective cationic channels, having a reversal potential quite sensitive to $K^+$ ions (in an unusual way). Even though its activation is facilitated by external hyperosmolarity, the osteoblast hyperpolarization-activated current differs from these currents both because it is carried by $Cl^-$ ions and because it is insensitive to $Gd^{3+}$. These two properties also separate the osteoblastic current from the smooth muscle current carried by stretch-sensitive hyperpolarization-activated channels which are cationic and $Gd^{3+}$ sensitive [28].

### Spontaneous Development of the Current

Our data showed that the high internal $Cl^-$ concentration that we used was partly responsible for the spontaneous development of the hyperpolarization-activated current (Fig. 1). However, it is not clear why the current often increased continuously for several tens of minutes after the internal $Cl^-$ concentration should have reached its maximum. Hypotheses such as slow removal of some inhibitory compound or slow $Cl^-$-induced destabilization of cytoskeletal structures possibly implicated in the mechanosensitivity of this current could be proposed.

The slow increase of the current studied after rupture of the cell-attached patch may also be related to the osmosensitivity of this current. The negative pressure applied inside the pipette to break the patch is released only after the initial membrane rupture, and thus is applied to the inside of the cell for at least a fraction of second. This transient negative pressure may have a mechanical effect similar to that of an external hyperosmolarity. However, it remains unclear why once in the whole-cell configuration with $K^+$-free solutions, the current studied usually reached an almost stable level, whereas the effect of changes in external osmolarity was always rapidly reversible.

Another phenomenon could partly explain the slow development of the current studied. After beginning cell dialysis, the slow diffusion of large intracellular molecules towards the pipette induces a slow decrease of the internal osmolarity towards that of the internal solution (slightly lower than the external osmolarity): this could affect the current in the same way as a slow increase in external osmolarity.

### Possible Role of the Activation of the $Cl^-$ Current by Hyperpolarization and by Mechanical Stimulation

At the present time, we cannot know whether the hyperpolarization-activated $Cl^-$ current of osteoblasts can be activated under physiological conditions. However,

possible modulation of these channels by temperature, intracellular messengers, intracellular anions and mechanical stimuli (likely to occur in bone) allow us to speculate that this is possible. This current could help these cells maintain membrane potential oscillations [23, 46]. Indeed, if a hyperpolarization below $E_{Cl}$ (due for example to the activation of $K^+$ channels), induced the activation of this $Cl^-$ current, the cell would repolarize towards $E_{Cl}$. The intracellular chloride concentration of some osteoblastic cells has been reported to be close to 35 mM [13]. Thus, repolarization towards $E_{Cl}$ could allow $Ca^{2+}$ entry (in particular through low-threshold T-type calcium channels), leading to an intracellular $Ca^{2+}$ concentration increase, which could again hyperpolarize the membrane by opening $Ca^{2+}$-activated $K^+$ channels. Such a pacemaker role has been clearly established for other types of hyperpolarization-activated currents, the cationic $I_f$ or $I_Q$ currents [20, 36].

It is tempting to propose that the osmosensitivity that we have detected experimentally by changing the osmotic pressure of the external solution could be important in bone during physiological mechanical stimulation under isosmotic conditions.

Other mechanosensitive ionic channels have been detected in osteoblastic cells by applying *negative* pressure to the external surface of the membrane in the cell-attached configuration [15, 16, 22]. The hyperpolarization-activated $Cl^-$ current, which could be partly responsible for a repetitive electrical activity, and could be activated by *positive* external pressure, could be one of the elements of mechano-electrical coupling in bone.

We wish to thank P. Ascher and B. Barbour for useful comments.

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