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# A VOLTAGE-DEPENDENT PROTON CURRENT IN CULTURED HUMAN SKELETAL MUSCLE MYOTUBES

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SUMMARY

1. A voltage-dependent proton current, $I_H$, was studied in cultured myotubes obtained from biopsies of human muscle, using whole-cell recording with the patch-clamp technique.

2. With a $pH_o$ of 8·0 and a calculated $pH_i$ of 6·3, $I_H$ was activated at voltages more depolarized than −50 mV and its conductance reached its maximum value at voltages more depolarized than +10 mV.

3. Studies of the reversal potential of $I_H$ during substitution of $K^+$, $Na^+$, $Ca^{2+}$, $Cl^-$, $Cs^+$ and $H^+$ in the extracellular solution indicated that protons were the major charge carriers of $I_H$.

4. $I_H$ was also activated during a voltage step to +22 mV with a $pH_o$ of 7·3 and a calculated $pH_i$ of 7·3.

5. Acidification of the extracellular solution led to a shift towards depolarized voltages of the conductance–voltage relationship.

6. Stationary noise analysis of $I_H$ suggested that the elementary event underlying $I_H$ was very small with a conductance of less than 0·09 pS.

7. Extracellular application of various divalent cations blocked $I_H$. The block by divalent cations was voltage dependent, being more efficient at hyperpolarized than at depolarized voltages. For $Cd^{2+}$, the Michaelis–Menten constant ($K_m$) for the block was 0·6 μM at −28 mV and 10·4 μM at +12 mV.

8. $Ca^{2+}$ was a less efficient blocker than $Cd^{2+}$ but could block $I_H$ at physiological concentrations (the $K_m$ values for the block were 0·9 mM at −38 mV and 7·3 mM at −8 mV).

9. The voltage-dependent properties of $I_H$ and its ability to be affected by pH and $Ca^{2+}$ suggest that $I_H$ might be used by skeletal muscle cells to extrude protons during action potentials.

10. A model of $I_H$ activation suggests that under extreme conditions, the conductance of $I_H$ can reach 40% of its maximum value after less than ten action potentials.

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## INTRODUCTION

Since its first description in snail neurones by Thomas & Meech (1982) a current carried by protons has been observed in several preparations (axolotl oocytes: Barish & Baud, 1984; neutrophils: Demaurex, Grinstein, Jaconi, Schlegel, Lew & Krause, 1993; see also Henderson, Chappel & Jones, 1987; Nanda & Grinstein, 1991; and rat alveolar epithelial cells: DeCoursey, 1991). It is believed that in these cells the proton current contributes to normal cellular functioning through the extrusion of protons and thereby participates in intracellular pH control.

During exercise, skeletal muscle cells may face acidic loads due to lactic acid production (Molé, Coulson, Caton, Nichols & Barstow, 1985; Hood, Schubert, Keller & Müller, 1988; Stainsby, Brechue & O'Drobinak, 1991; Roth, 1991). A typical change of intracellular pH during intense exercise is from 7·0 to 6·5, although values as low as 5·9 have been reported (Mainwood & Renaud, 1984; Kowalchuk, Heigenhauser, Lindinger, Sutton & Jones, 1988). Low pH alters the performance of the tension-producing mechanism (Mainwood & Renaud, 1984; Westerblad, Lee, Lännergren & Allen, 1991) and blocks the enzyme phosphofructokinase (Trivedi & Danforth, 1966), so that acidification can lead to reduced muscle tension. Three acid–base species transporters, however, seem to limit intracellular acidification in skeletal muscle: the $Na^+$–$H^+$ and the $Cl^-$–$HCO_3^-$ exchangers (Aickin & Thomas, 1977) and the lactate transporter (Mason & Thomas, 1988). Westerblad & Allen (1992) concluded that in mouse muscle the lactate transporter was the most important pH controlling mechanism during a fatigue-producing effort. In their experimental conditions, the lactate transporter appeared to account for the required proton extrusion power.

The goal of the present work was to see whether a proton current existed in human muscle membrane and if so to investigate the conditions that may affect its physiological functioning. In the present work, we describe a proton current, $I_H$, in cultured human myotubes, which is similar in many respects but not identical to the proton currents already described in other cell types. A proton current has never been characterized electrophysiologically in skeletal muscle fibres, although its presence has been discussed in moth and frog skeletal muscle (Rheuben, 1972; Lynch, 1985). We show that $I_H$ can be affected by extracellular pH and calcium, and that the block by divalent cations is voltage dependent. Also, using variance analysis of current noise, we confirm the previous observation made in invertebrate neurones (Byerly & Suen, 1989) that the elementary event underlying $I_H$ is extremely small. We discuss the possibility that $I_H$ in muscle has the properties required to extrude protons during action potentials.

## METHODS

### *Dissociation and culture procedures*

Muscle biopsies (50–490 mg) were obtained during corrective orthopaedic surgery of three patients between 6 and 11 years old without any known neuromuscular disease. The procedure was done in accordance with the guidelines of the ethical committee of the University Hospital in Geneva (Switzerland).

The dissociation procedure to isolate and prepare clonal cultures of satellite cells was as previously described (Baroffio, Aubry, Kaelin, Krause, Hamann & Bader, 1993). Briefly, the

muscle sample was cleaned to remove fat and connective tissues, minced and placed at 37 °C for 1 h in a solution containing 0·05 % trypsin and 0·02 % EDTA under continuous agitation. The cells were then centrifuged and resuspended several times in wash medium (Ham's F10 supplemented with 15 % fetal calf serum) in order to pellet muscular debris. Tris–ammonium chloride buffer was added to lyse red blood cells. Individual satellite cells (clonal culture) were manually collected with a micropipette under a microscope and cultured first in proliferation medium (Ham's F10 supplemented with 15 % fetal calf serum; bovine serum albumin, 0·5 mg $ml^{-1}$; fetuin, 0·5 mg $ml^{-1}$; epidermal growth factor, 10 ng $ml^{-1}$; dexamethasone, 0·39 $\mu$g $ml^{-1}$; insulin, 0·18 mg $ml^{-1}$; and gentamycin, 0·1 $\mu$g $ml^{-1}$) in which satellite cells were actively proliferating for a period of 30 to 165 days, and then in a differentiation medium (DMEM supplemented with bovine serum albumin 0·5 mg $ml^{-1}$; epidermal growth factor, 10 ng $ml^{-1}$; insulin, 10 $\mu$g $ml^{-1}$; and gentamycin, 1 $\mu$g $ml^{-1}$) in which satellite cells fused to form myotubes. Myotubes were then kept in differentiating medium for periods varying between 7 and 96 days before electrophysiological recordings were performed. One day before recording, myotubes were treated with 0·05 % trypsin (0·02 % EDTA) and replated at low density. This enzyme treatment and the mechanical trituration yielded spherical myotubes which facilitated the patching procedure.

*Electrophysiological recordings*

Currents were recorded under voltage clamp in the whole-cell configuration of the patch technique using either a List EPC-7 or an Axopatch 200 amplifier (Fig. 8*A*). Patch pipettes were made from borosilicate glass using a BB-CH puller (Mecanex, Switzerland) and coated with Sylgard when current noise was studied. The resistance of the pipettes was between 3 and 5 MΩ. If not specified, currents were recorded at 20–22 °C, sampled at 250 Hz and low-pass filtered at 1 kHz. All potentials in the text and figures are corrected for junction potentials. The reference electrode to measure junction potentials was either a pipette filled with a 3 M KCl solution or an agar bridge containing 3 M KCl. Series resistance compensation was maximally used during the experiments described in Fig. 8*A*. Capacitance transients were masked in the figures. Acquisition, analysis and graphical representation of data were performed with a program written by us.

*Series resistance and size of myotubes*

The resistance between the input connector of the head stage and the cytosol of the myotubes (series resistance) and the size of the myotubes were evaluated from a capacitive transient elicited by a voltage step to −90 mV from a holding potential of −80 mV. As the capacitance of the membrane was charged during the voltage step, the current injected by the amplifier was exponentially decaying. The series resistance compensation circuit of the amplifier was not used during these measurements. The series resistance was evaluated from the maximum initial current injected at the beginning of the 10 mV voltage step (extrapolation at $t = 0$ of a fitted theoretical equation: $I = I_{max} \exp(-t/\tau)$). In eighty-one randomly selected recordings, the access resistance was $8 \pm 0{\cdot}4$ MΩ. The membrane capacitance of the myotube was obtained by dividing the time constant of the exponentially decaying current by the series resistance. The membrane capacitance calculated from the area under the capacitive transient gave similar results. The average capacitance of eighty-one myotubes was $182 \pm 9$ pF which corresponds to spherical cells of $76 \pm 2$ $\mu$m in diameter using a specific membrane capacitance of 1 $\mu$F $cm^{-2}$. Results are expressed as the means ± S.E.M.

*Solutions and materials*

Between recordings, cells were perfused with a medium containing (mM): NaCl, 150; KCl, 5; $CaCl_2$, 5; $MgCl_2$, 2; Hepes, 5; pH adjusted to 7·3. When $K^+$ currents were recorded, the intracellular solution contained (mM): KCl, 150; EGTA, 10; $CaCl_2$, 9·1; Hepes, 5; $MgCl_2$, 1; pH 7·3; extracellular solution contained (mM): choline, 150; KCl, 5; $MgCl_2$, 5; Hepes, 5; pH 7·3.

Tables 1 and 2 list the various intra- and extracellular solutions that were used when recording $I_H$. In most experiments, the extracellular solution contained aspartate. As aspartate is known to chelate divalent cations (Martell & Smith, 1974), free divalent cation concentrations were computed according to Perrin & Sayce (1967).

Trypsin (from bovine pancreas) and ethylenediaminetetracetic acid disodium salt (EDTA) were from Boehringer Mannheim, Germany. Ham's F-10, Dulbecco's modified Eagle's medium (DMEM), and gentamycin were from Gibco, Basel, Switzerland. Bovine serum albumin (BSA), dexamethasone, fetuin, insulin, 2-(*N*-morpholine) ethanesulphonic acid (Mes), *N*-2-

hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), cyano-4-hydroxycinnamic acid (cinnamate), L-aspartic acid, D-gluconic acid, nifedipin, and choline chloride were from Sigma Chemical Co, Switzerland. Epidermal growth factor (EGF) was from Collaborative Research, Bedford, MA, USA. Fetal calf serum (FCS) was from Ready System, Bad Zurzach, Switzerland. Tris(hydroxymethyl)aminomethane (Tris), $CuCl_2$, $BaCl_2$, $CaCl_2$, $MgCl_2$, $ZnCl_2$, NaCl, KCl, KOH,

TABLE 1. Composition of intracellular solutions (mM)

| Soln. | pH | Buffer (100 mM) | CsOH | Aspartic acid | CsCl | $MgCl_2$ | EGTA or BAPTA |
|---|---|---|---|---|---|---|---|
| A | 5·5 | Mes | 110 | 90 | 0 | 1 | 0·5 |
| B | 7·0 | Hepes | 112 | 88 | 0 | 1 | 0·5 |
| C | 5·5 | Mes | 89 | 69 | 20 | 1 | 0·5 |

TABLE 2. Composition of extracellular solutions (mM)

| Soln | pH | Hepes | Tris | NMG | Aspartic acid | HCl | $MgCl_2$ |
|---|---|---|---|---|---|---|---|
| 1 | 7·3 | 100 | 0 | 119·4 | 80·7 | 0 | 1 |
| 2 | 8·0 | 100 | 0 | 138 | 62 | 0 | 1 |
| 3 | 8·0 | 10 | 0 | 148·8 | 141·2 | 0 | 1 |
| 4 | 8·0 | 0 | 100 | 78 | 122 | 0 | 1 |
| 5 | 7·4 | 100 | 0 | 125·6 | 0 | 81·4 | 1 |

and HCl were from Merck, Zurich, Switzerland. *N*-methyl-D-glucamine (NMG), ethylenediaminetetracetic acid (EGTA), 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), magnesium D-gluconate, $CdCl_2$, CsCl, and tetraethylammonium chloride (TEA-Cl) were from Fluka, Buchs, Switzerland.

## RESULTS

An outward current was studied in 130 human myotubes (average capacitance 182 pF, see Methods) coming from five different clones. Clonal cultures were prepared to exclude the possibility of recording from non-muscle cells. Non-permeant ions were applied to the intra- and extracellular solutions in order to eliminate the contribution of $K^+$, $Na^+$, $Ca^+$ and $Cl^-$ currents.

### *Presence of an outward current in muscle myotubes*

An outward current was activated when a myotube was stepped to a series of depolarized voltages between −70 and +30 mV from a holding potential of −78 mV (Fig. 1*A*). Figure 1*B* shows that the current was activated at potentials more positive than −50 mV and that the conductance reached a maximum near +20 mV. Extracellular pH was 8·0 and the pH of the solution in the patch electrode was 5·5. The relationship between the voltage and the conductance was adequately described by a Boltzmann equation. In eight cells the maximum conductance was $7·3 \pm 1·5$ nS ($49 \pm 9$ pS/pF) and the voltage at half-maximum conductance was $-30 \pm 3$ mV. The gating charge calculated from the fitted Boltzmann equation was $2·4 \pm 0·2$ elementary charges. As we shall see below, the outward current is carried by protons and a gating charge of +1·8 elementary charges was found for proton currents recorded from *Ambystoma* oocyte (Barish & Baud, 1984).

Figure 1*C* illustrates the behaviour of the outward current during a sustained depolarization at −8 mV. In three cells, during a period of more than 2 min the amplitude of the current decreased by 6±3%. Measurements of the reversal potential of the current at various times after its activation suggested that this

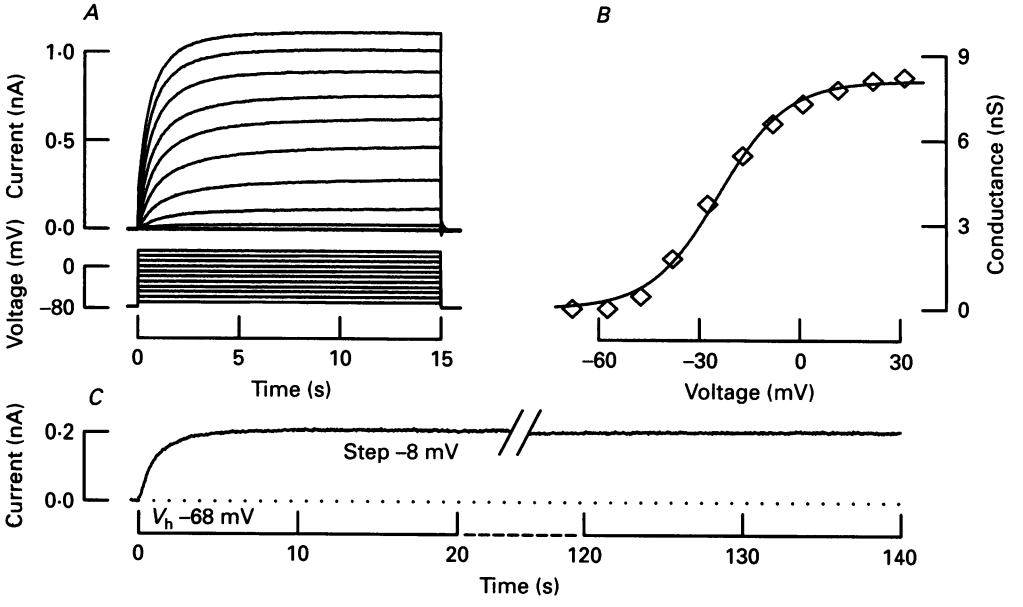


Fig. 1. Voltage-dependent properties of the outward current. *A*, a myotube was held steadily at −78 mV and stepped to a series of depolarized voltages during 15 s. Current traces are not corrected for leak current. Recording was done with intracellular solution A and extracellular solution 2. *B*, the leak-subtracted current was measured at the end of the step and divided by $V_s - E_r$ ($V_s$ is the voltage during a step and $E_r$ the reversal potential of $I_H$ in this cell which was −93 mV) to compute the steady-state conductance; the conductance was plotted as a function of $V_s$. Leak current was estimated by linear extrapolation of the current recorded at −88, −78 and −68 mV. The continuous line is a Boltzmann equation $g(V) = g_{max}/[1+\exp(Q(V-V_0)/kT)]$ where $g$ is conductance, $k$ is the Boltzmann constant and $T$ is absolute temperature. The fit gave $Q = 2{\cdot}53$ elementary charges and $V_0 = -24{\cdot}6$ mV. *C*, another myotube was held at −68 mV and stepped to −8 mV for 140 s, to show that there was no voltage-dependent inactivation of the leak-subtracted current. Recording was done in solution 2; intracellular solution was solution A.

decrease was due to a reduction of the driving force for protons as a result of the standing outward flow of protons (data not shown; see DeCoursey, 1991, for a discussion of this effect).

In order to examine whether protons were the charge carriers of the outward current, the reversal potential of the current was measured at two different extracellular pH values, near 8·0 and 7·3 (Fig. 2). The current was first activated by stepping the voltage from −68 to +22 mV and, subsequently, the level of the voltage was set to a series of hyperpolarized values where relaxation currents were measured (Fig. 2*A*). The same procedure was repeated after the application of $Cd^{2+}$, which nearly completely suppressed the current (traces labelled $Cd^{2+}$). The difference

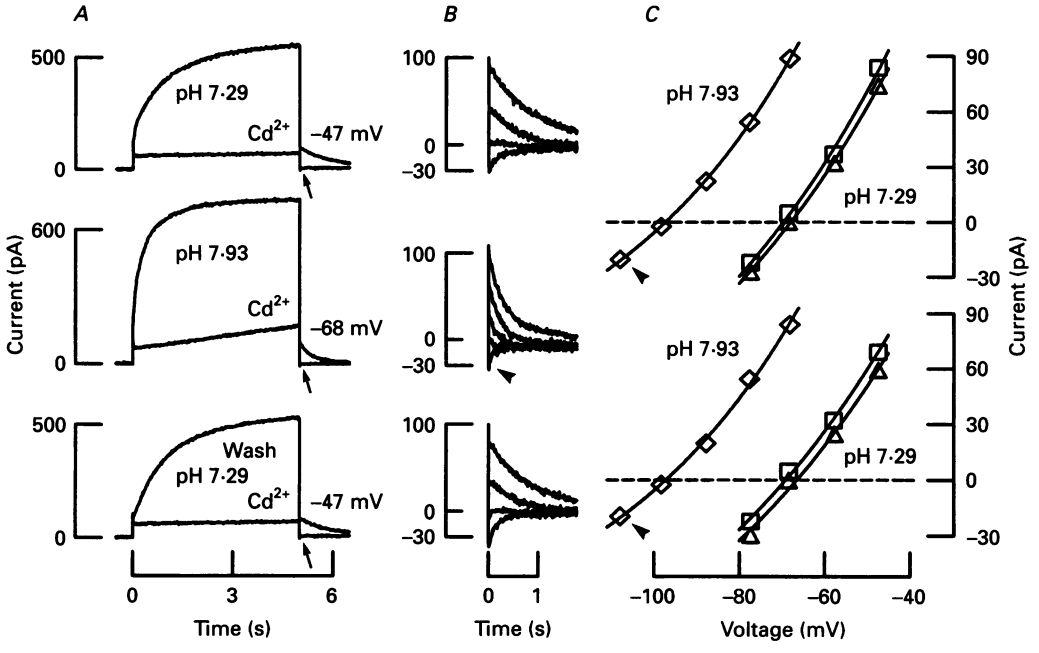


Fig. 2. The reversal potential of the outward current depends upon the transmembrane pH gradient. *A*, a myotube was held steadily at −68 mV and the voltage was stepped to +22 mV to activate the current. Then, the level of the voltage was set to a series of hyperpolarized values where relaxation currents could be measured. Two relaxation currents are illustrated for each set of data. For the top panel, a relaxation at −47 mV and another at −68 mV (the reversal potential) are shown. This first series of recordings in the top panel was done in solution 1 (which had for this experiment a precise pH of 7·29) and the intracellular solution was solution A. The same procedure was repeated after the application of $Cd^{2+}$ (solution 1 supplemented with 260 μM free $Cd^{2+}$; total $Cd^{2+}$ = 3 mM), which nearly completely suppressed the voltage-dependent outward current (trace labelled $Cd^{2+}$). *B*, differences at each relaxation voltage between the current traces measured in the presence and in the absence of $Cd^{2+}$ are illustrated. The value of the difference between the relaxation current measured at the arrow in the presence and in the absence of $Cd^{2+}$ was plotted as a function of the voltage during relaxation in the upper panel of *C* (□). The extracellular solution was then switched to a more basic solution (solution 2, which had a precise pH of 7·93) and the entire procedure was repeated (free $Cd^{2+}$ was 40 μM; total $Cd^{2+}$ = 3 mM). Relaxation currents are illustrated at −68 and −98 mV (the new reversal potential). (The arrowheads indicate the tail current trace at −100 mV for which the current in the presence of $Cd^{2+}$ was not available, as the cell was lost during recording at this potential. Therefore, the $Cd^{2+}$ trace at this voltage was linearly extrapolated from the current trace recorded immediately before at −100 mV, in the presence of $Cd^{2+}$.) The difference between the relaxation current amplitudes measured at the arrow in the presence and in the absence of $Cd^{2+}$ was again plotted in the upper panel of *C* (◇); the extracellular solution was changed again to solution 1 (recovery, pH 7·29) and the entire procedure repeated (△). In the lower panel of *C*, the currents represent the difference between the amplitude of the relaxation currents measured at the arrows in *A* and the currents measured 1·8 s after the beginning of relaxation. Symbols are as in the upper panel. This simple method of measuring the reversal potential was generally used, as it gave the same result as the method illustrated in the upper panel. The continuous lines in *C* are Goldman–Hodgkin–Katz equations (see Hille, 1992, chap. 13). Equations were fitted by allowing the fitting procedure to adjust the permeability and the intracellular proton concentration.

between the two relaxation current traces recorded in the absence and presence of $Cd^{2+}$ is illustrated in Fig. 2*B*, together with the corresponding differences recorded at three other relaxation voltages. The amplitude of the difference between the relaxation current measured at the arrow in the presence and absence of $Cd^{2+}$ was plotted in the upper panel of Fig. 2*C* (□) as a function of the voltage during relaxation. In the lower panel of Fig. 2*C*, the squares represent the difference between the amplitude of the relaxation current measured at the arrow and 1·8 s after the beginning of relaxation. The result of the analysis indicated that this simpler method gave the same results as the $Cd^{2+}$ block and it was used to analyse most of our other experiments on reversal potentials.

The shift of the reversal potential was in the direction expected for a current carried by protons and the effect of the pH change was reversible (△, Fig. 2*B*). In eight cells, the shift of the reversal potential produced by a pH change from 7·3 to 8·0 was $29 \pm 3$ mV, i.e. smaller than the 41 mV shift predicted by the Nernst equation for this extracellular pH change.

Thus, either there is another ion contributing to the outward current or the intracellular pH underneath the membrane is itself affected by the change in extracellular pH.

In order to determine whether the major charge carriers of the outward current were protons and also to be sure that protons were not moving through other types of voltage-gated channels, a series of experiments was done in which the effects on the current of various ions were examined. The changes in reversal potential of the outward current induced by ion substitutions are plotted in Fig. 3.

Figure 3 shows that none of the various ion substitutions ($Na^+$, $K^+$, $Cl^-$, $Ca^+$ and $Cs^+$) had a marked effect on the reversal potential of the current (for details, see legend of Fig. 3). The permeability of $Cs^+$ ions was tested because $Cs^+$ was used routinely at a high concentration in the intracellular solutions in order to block $K^+$ currents. Regarding $Na^+$, in one additional experiment not included in Fig. 3, a hundred-fold increase of extracellular $Na^+$ from 1·38 mM to 138 mM shifted the reversal potential of the outward current by −3 mV. The addition of sodium to the extracellular solution induced the appearance of a transient inward current at the beginning of a depolarizing step, but this current inactivated within a few milliseconds.

It is important to note that both intra- and extracellular concentrations of protons were in the micromolar range and that in many cases the driving force for the ion tested was very large in the experiments illustrated in Fig. 3. Therefore, even a very small permeability ($P_X/P_{proton} < 10^{-6}$) for an ion X could have evoked a shift of a few millivolts in the reversal potential of the outward current.

As there were rather high concentrations of pH buffers on both sides of the membrane, the possibility that a pH buffer could be a charge carrier had to be considered. A first test was to change the extracellular Hepes concentration by a factor of ten. This manipulation would have changed the reversal potential of the outward current by 58 mV if Hepes had been the charge carrier. Although the amplitude of the outward current was, in two cells, reduced by 23 and 32% when extracellular Hepes was reduced, the reversal potential of the current was not markedly affected: the observed shifts were 2 and 5 mV. In a second set of

experiments all extracellular Hepes was substituted by Tris and there was no significant modification of the reversal potential of the outward current.

Although ionic substitutions strongly suggested that protons do not move through a conventional ionic channel, several drugs were tested to confirm this result. We

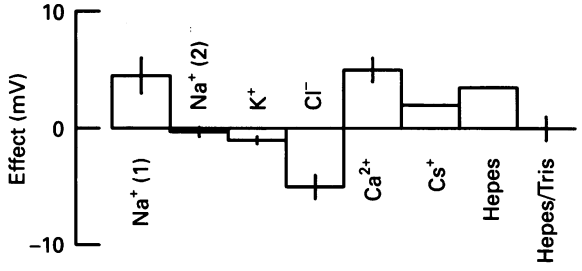


Fig. 3. Effect of various ions on the reversal potential of the outward current. The reversal potential of the outward current was evaluated as described in Fig. 2*B* (lower panel). $Na^+$ (1): an increase of extracellular $Na^+$ from 10 to 50 mM at pH 7·30 (solution 1 in which 50 mM NMG was substituted by either 10 mM NaOH + 40 mM NMG or 50 mM NaOH) shifted by $+5 \pm 2$ mV the reversal potential of the current ($n = 4$). $Na^+$ (2): a decrease of extracellular $Na^+$ from 50 to 10 mM at pH 7·92 (solution 2 in which 50 mM NMG was substituted by either 50 mM NaOH or 10 mM NaOH + 40 mM NMG) shifted the reversal potential by $-0{\cdot}3 \pm 0{\cdot}4$ mV ($n = 3$); the intracellular solution was solution A in which 10 mM CsOH was substituted by 10 mM NaOH. $K^+$: when extracellular $K^+$ was raised from zero (solution 2 supplemented with 3 mM NMG-Cl) to 3 mM (solution 2 supplemented with 3 mM KCl) the reversal potential shifted by $-1{\cdot}0 \pm 0{\cdot}3$ mV ($n = 3$); CsCl (10 mM) was present in both solutions to block inward rectifier $K^+$ current; intracellular solution was solution A. $Cl^-$: an increase of extracellular $Cl^-$ from 2 mM (solution 2) to 64 mM (substitution of 62 mM aspartic acid by HCl) shifted the reversal potential by $-5 \pm 1$ mV ($n = 5$); intracellular solution was solution A. $Ca^{2+}$: when 10 mM $MgCl_2$ of the extracellular medium (solution 2 in which 9 mM NMG-aspartate was replaced by 9 mM $MgCl_2$) was substituted by an equal concentration of $CaCl_2$ the reversal potential shifted by $+5 \pm 1$ mV ($n = 4$); intracellular solution was solution C; note that when the effect of $Ca^{2+}$ was tested, the depolarizing steps did not exceed $-20$ mV, in order to minimize activation of calcium and calcium-activated currents. $Cs^+$: in 2 cells, extracellular $Cs^+$ was raised from zero (solution 2) to 109 mM (solution 2 in which 109 mM NMG was substituted by an equal concentration of CsOH); the reversal potential of $I_H$ was shifted by $+2$ mV in both cells; intracellular solution was solution C. Hepes: a decrease of extracellular Hepes from 100 mM (solution 2) to 10 mM (solution 3) shifted, in 2 cells, the reversal potential by $+2$ and $+5$ mV. Hepes/Tris: complete substitution of extracellular Hepes (solution 1) by Tris (solution 4) shifted the reversal potential by $0 \pm 1$ mV ($n = 3$); intracellular solution was solution C.

eliminated the possibility of protons moving through L-type $Ca^{2+}$ channels by using the dihydropyridine antagonist nifedipin. In five cells, $98 \pm 2\%$ of the outward current remained after bath application of 1 $\mu$M nifedipin. We think that protons do not move through DHP-insensitive $Ca^{2+}$ channels for several reasons; the sensitivity of the proton current to $Cd^{2+}$, its insensitivity to amiloride (data not shown) and the absence of inactivation properties of the proton current exclude movement through a low-threshold $Ca^{2+}$ current. Recently, an N-type DHP-insensitive $Ca^{2+}$ current has been described in human myotubes (Rivet, Cognard, Imbert, Rideau, Duport & Raymond, 1992). Unlike the proton current, however, its threshold for activation is more depolarized than $-20$ mV and it inactivates strongly within a second.

Can we eliminate the possibility of protons moving through potassium channels? We did not see any change in amplitude of the outward current during a depolarizing step after extracellular application of 3 mM $K^+$, which should have produced an inward current. On the other hand, TEA (10 mM) blocked a proportion ($35 \pm 15\%$, $n = 5$) of the outward current. Movement of protons through TEA-sensitive $K^+$ channels seemed unlikely, however, since we observed that $Cd^{2+}$ was a potent blocker of the outward current at micromolar concentrations (see also below) but not of the $K^+$ current. Indeed, the TEA (20 mM)-sensitive outward current of myotubes, which is carried mainly by $K^+$ current, was reduced by only $10 \pm 8\%$ ($n = 4$) in the presence of 3 mM $Cd^{2+}$ (data not shown; recording with $K^+$ instead of $Cs^+$ in the intracellular solution, see Methods). Considering our various experiments on the effect of $K^+$ and $K^+$ blockers, we conclude that if protons moved through $K^+$ channels, these channels should have the following, unusual characteristics: (i) they should behave as perfect diodes, not allowing inward movement of $K^+$ (see our experiment on $K_o^+$ and reversal potential); (ii) they should be insensitive to 100 mM intracellular $Cs^+$; (iii) they should be permeable to $H^+$, which, unlike $K^+$, should be able to move inward and outward; (iv) they should be sensitive to $Cd^{2+}$ at micromolar concentrations; and (v) they should be weakly sensitive to TEA.

Thus, our experiments lead us to conclude that protons are the major charge carriers of the outward current, that $Na^+$, $K^+$, $Cl^-$, $Ca^+$, and $Cs^+$ do not contribute significantly to the outward current and that this current does not originate from proton flux through conventional voltage-gated ionic channels. We shall call this current $I_H$. We explain the discrepancy between the predicted and observed shift of the reversal potential of $I_H$ mentioned above by a lack of control of the intracellular pH underneath the membrane. Incomplete control of intracellular pH was reported in very small cells (3–5 pF) in which the pH was measured with a pH-sensitive dye (Demaurex *et al.* 1993). Assuming that the outward current is a pure proton current, and that the $pH_o$ near the membrane is that of the bulk solution, the reversal potential values in Fig. 2 would correspond to an intracellular pH of 6·1 when the extracellular pH is 7·29 and of 6·26 when the extracellular pH is 7·93. Note that the pH of the solution in the patch electrode was 5·5.

In the experiments described above, the choice of the intracellular pH was dictated by the objective of maximizing the driving force of the proton current, in order to facilitate its visualization. Although the low intracellular pH values used here were within the physiological range and could be reached during an exhaustive anaerobic exercise (Hood *et al.* 1988), we wondered whether $I_H$ could also be activated at resting intracellular pH. The experiment illustrated in Fig. 4 shows that this is the case. In this experiment, the pH of the solution in the patch electrode was 7·00. In Fig. 4*A*, the continuous current traces represent the net outward current during a step to +22 mV at extracellular pH 8·0 and 7·33. The dotted current trace is the current during a step to +41 mV at extracellular pH 7·33, demonstrating that a conductance in excess of 2 nS can be reached under these pH conditions. The relaxation currents illustrated are flat because they were recorded at voltages in the vicinity of the reversal potential (−32 mV at pH 8·0 and −2 mV at pH 7·33, Fig. 4*B*). Thus, this experiment shows that a significant outward proton current can be activated at resting intra- and extracellular pH values.

*The conductance–voltage relationship of $I_H$ is affected by extracellular pH*

In several preparations (Barish & Baud, 1984; Bylerly, Meech & Moody, 1984; see also DeCoursey, 1991), extracellular acidification shifts the conductance–voltage relationship along the voltage axis and this also occurs in myotubes.

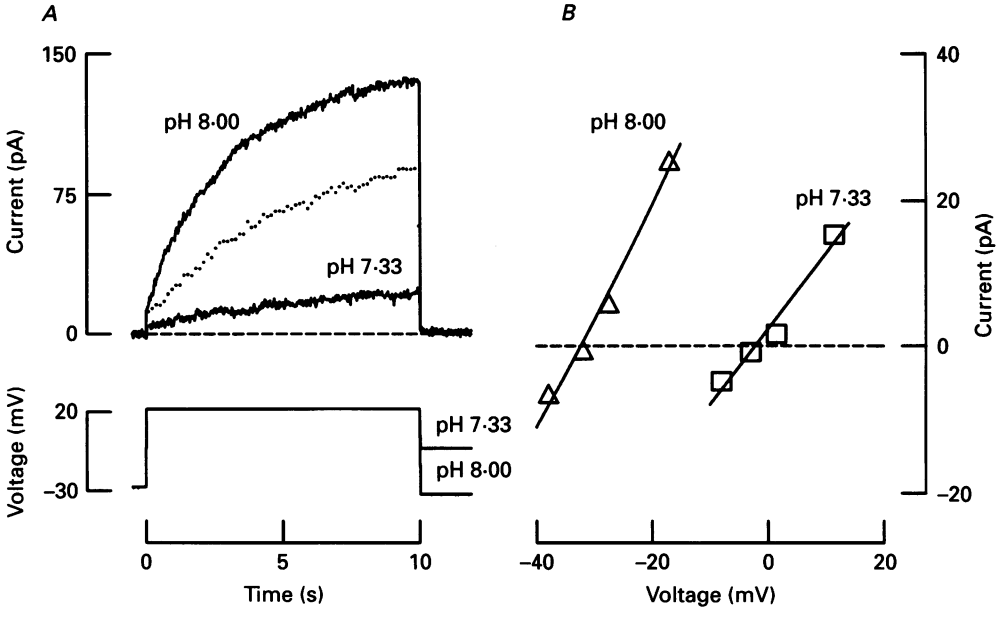


Fig. 4. $I_H$ can be recorded at resting pH. *A*, a myotube was held steadily at −28 mV and the voltage was stepped to +22 mV in order to activate $I_H$; then, the voltage was returned to near the reversal potential of the current, i.e. −2 mV at $pH_o$ 7·33 (solution 1), or −35 mV at $pH_o$ 8·00 (solution 2). *B*, the relaxation currents were measured as in Fig. 2 and plotted as a function of voltage (□ at $pH_o$ 7·33; △ at $pH_o$ 8·00); continuous lines are Goldman–Hodgkin–Katz equations fitted to the data as in Fig. 2; currents are leak-subtracted (leak current is the current remaining after application of 3 mM $Cd^{2+}$ in the external medium; free $Cd^{2+}$ concentration was 40 μM at pH 8·00 and 260 μM at pH 7·33); intracellular solution was solution B. The dotted line in *A* corresponds to the current recorded at pH 7·33 during a step to +42 mV.

Figure 5 illustrates the result of an experiment in which the maximum conductance of $I_H$ was measured at a series of voltages and at two different extracellular pH values. It can be seen that a decrease in extracellular pH shifts the conductance–voltage relationship to the right. In three cells, the voltage at half-activation was shifted by +15±2 mV when the extracellular pH was changed from 8·0 to 7·3, and there was a non-significant increase in the maximum conductance (+2±4%). The maximum conductance increased at acidic extracellular pH values in *Ambystoma* oocyte (Barish & Baud, 1984) but decreased under similar conditions in snail neurones (Byerly *et al.* 1984).

An experiment like that illustrated in Fig. 5 could theoretically give a clue as to whether $I_H$ is carried by protons leaving the cell or $OH^-$ (or $HCO_3^-$) entering the cell (DeCoursey, 1991). If $OH^-$ (or $HCO_3^-$) was the charge carrier, a reduction of

extracellular pH, which corresponds to a reduction of $OH^-$ (or $HCO_3^-$) concentration, might lead to a limitation of the maximal amplitude of the current during very depolarized voltage steps, due to a depletion of $OH^-$ (or $HCO_3^-$) near the membrane. Figure 5 illustrates that neither at extracellular pH 8·0 nor at pH 7·3 is there a

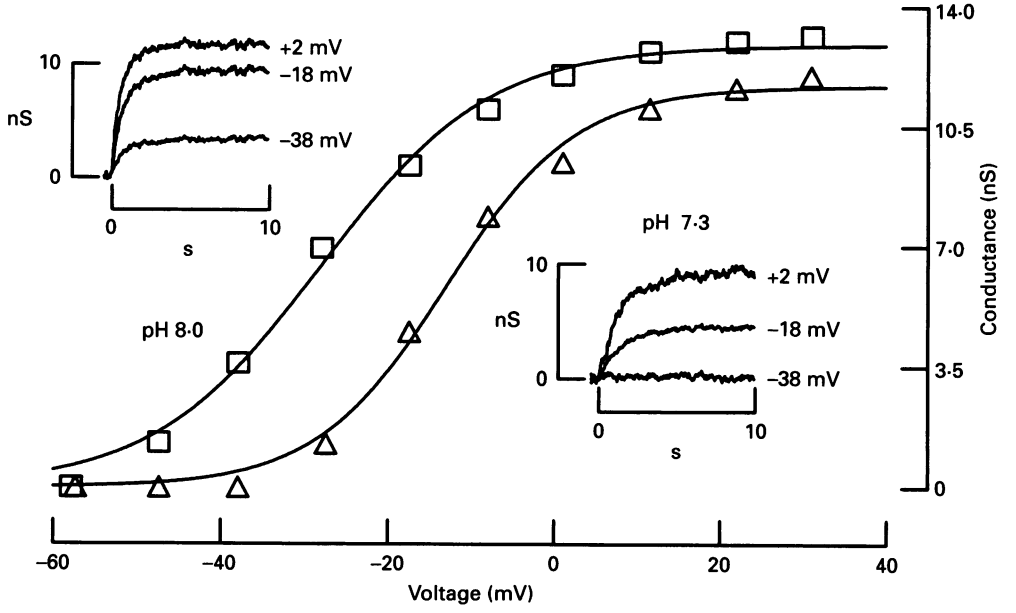


Fig. 5. Extracellular pH affects $I_H$. A myotube was held at −68 mV and depolarized for 12 s to various potentials every 24 s. $I_H$ was recorded in extracellular solution 2 (pH 8·0; ○) and extracellular solution 1 (pH 7·3; △). Intracellular solution was solution A. Currents were measured at the end of the depolarizing step, corrected for leak current (obtained by linear extrapolation of the current recorded during a 10 mV depolarization from −68 mV), and divided by $V_s - E_r$ ($V_s$ is the voltage during a step and $E_r$ the reversal potential of −75 mV at pH 8·0 and −45 mV at pH 7·3) to compute the steady-state conductance. Continuous lines are Boltzmann equations with a $V_0$ of −28 mV and $Q$ of 2·84 at pH 8·0 and a $V_0$ of −13 mV and $Q$ of 3·01 at pH 7·3. Insets represent leak-subtracted current traces recorded at pH 8·0 and 7·3 during voltage steps to −38, −18 and +2 mV.

tendency for the steady-state conductance to decrease at the largest depolarizations studied. This implies that the currents do not show any sign of saturation at either pH values during depolarizations up to +32 mV. Although this observation would be consistent with protons rather than $OH^-$ (or $HCO_3^-$) being the charge carrier of the current, it certainly does not allow us to exclude the anionic acid–base species as charge carriers. To simplify the descriptions of the outward current studied here, we considered that it was carried by protons rather than $OH^-$ or $HCO_3^-$.

*Can the elementary proton current be recorded?*

In an attempt to answer this question, stationary noise analysis was performed, as $I_H$ does not inactivate for some minutes during a sustained depolarization (Fig. 1*C*). Firstly, a myotube was stepped from a holding voltage of −68 to −48 mV, to

confirm the presence of $I_H$ (Fig. 6*A*, lower current trace). Then, the voltage was maintained steady at −48 mV and the current was recorded at a high sampling rate (10 kHz) and with the low-pass filter set at 5 kHz. Samples of current were recorded every second, and an example is illustrated at the top of Fig. 6*A*. The same procedure

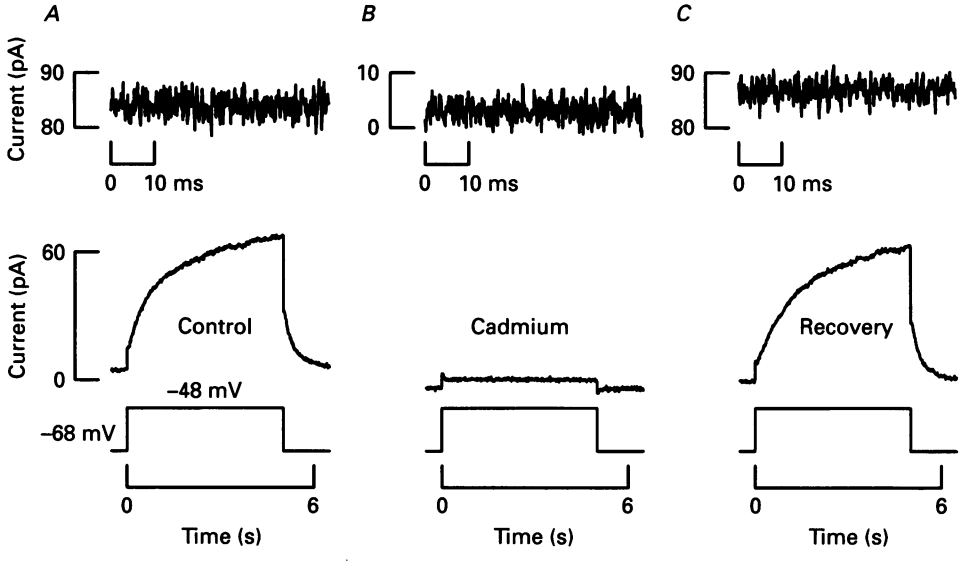


Fig. 6. Effect of $Cd^{2+}$ on $I_H$ noise. A, a myotube was held steadily at −68 mV and stepped to −48 mV for 5 s in the presence of solution 2 (intracellular solution was solution A). This voltage step activated $I_H$ but a steady level was not reached. The voltage was then continuously set to −48 mV and an example of current recording taken at high resolution is illustrated (sampling 10 kHz, filter 5 kHz) at the top of the figure on a fast time scale. Note that the mean steady current is 85 pA. In *B*, the same procedure was repeated in the presence of 40 $\mu$M free $Cd^{2+}$ (mean steady current is 3 pA) and in *C*, recordings after recovery in solution 2 are illustrated.

was repeated in the presence of $Cd^{2+}$ (Fig. 6*B*) and it can be seen that $I_H$ was totally suppressed by $Cd^{2+}$ during the voltage step. The current recorded at high resolution in the presence of $Cd^{2+}$ is illustrated at the top of Fig. 6*B*. Finally, $Cd^{2+}$ was removed and the procedure was repeated (Fig. 6*C*).

Visual inspection of the current noise traces showed that there was little difference in the peak-to-peak amplitude of the steady current noise recorded in the absence or in the presence of $Cd^{2+}$. This impression was confirmed by computing the variance of the steady current in the three conditions. Fifteen samples of current lasting 100 ms were used. In the control condition, $\sigma^2$ was 3·5 $pA^2$; it decreased to 3·3 $pA^2$ in the presence of $Cd^{2+}$, and returned to 3·4 $pA^2$ after $Cd^{2+}$ was washed out.

This experiment allowed us to set an upper limit for the size of the elementary $I_H$, on the basis of the relationship between the elementary current ($i_H$), the variance of the macroscopic current noise ($\sigma^2$), and the mean macroscopic current ($I_H$):

$$i_H = \sigma^2/I_H(1-p).$$

We considered that the probability of the channel being open ($p$) was negligible

as $p$ is low at $-48$ mV (see Fig. 1*B*). If we assume that the variance suppressed by $Cd^{2+}$ (0·2 $pA^2$) corresponds to the proton current, $i_H$ is 2 fA (equivalent to 0·05 pS). In four cells, the mean elementary conductance calculated from the macroscopic current noise suppressed by $Cd^{2+}$ was $0·09 \pm 0·05$ pS. Note that even if we assumed that the entire variance observed under control conditions was due to $I_H$ (which is not so, as most of it was insensitive to $Cd^{2+}$) $i_H$ would still be very small (43 fA, equivalent to 0·85 pS). These observations confirm those made in snail neurones (Byerly & Suen, 1989), suggesting that the elementary proton current was very small and undetectable with the currently available techniques. It is worth noting that the low concentration of protons in both intra- and extracellular solutions could by itself explain a very small elementary current (see Hille, 1992, chap. 11).

Does $I_H$ flow through an ionic channel or is the current generated by an electrogenic transport mechanism? This question cannot be definitely answered as the elementary proton current could not be resolved, and as the existence of voltage-dependent properties or of a reversal potential do not exclude a transporter. In addition, ATP-dependent proton transporters are able to carry current as large as $I_H$ (Hedrich, Kurkdjian, Guern & Flügge, 1989). It is unlikely, however, that ATP is needed to generate or maintain $I_H$, as $I_H$ was recorded in the absence of ATP in the intracellular solution. Also, no run-down was observed during recordings lasting for more than 1 h.

*Voltage-dependent block of $I_H$ by divalent cations*

Block of currents by divalent cations carried by protons has been reported in several preparations (Byerly *et al.* 1984; Barish & Baud, 1984; Meech & Thomas, 1987; Byerly & Suen, 1989; DeCoursey, 1991; Demaurex *et al.* 1993). It was shown above (Fig. 2) that in cultured muscle, $Cd^{2+}$ was a potent blocker of $I_H$. The potency of a series of divalent cations was examined in a total of eight cultured human myotubes during step-depolarizations to $-28$ mV from a holding voltage of $-68$ mV. There were two reasons for choosing this particular level of voltage step: firstly, inaccuracies caused by currents carried by divalent ions through the calcium channels during the testing of $Ba^{2+}$ and $Ca^{2+}$ were minimized (Rivet *et al.* 1992), and secondly, as is shown below, the block by divalent cations is voltage dependent and the voltage step chosen was a compromise between a fair level of activation of $I_H$ and minimum relief of the block by depolarization. The results suggest that the order of potency of the divalent cation is $Cu^{2+} > Zn^{2+} > Cd^{2+} > Ca^{2+} = Ba^{2+} > Mg^{2+}$. This result is similar to that described by Byerly & Suen (1989) in snail neurones.

The blocking effects on $I_H$ of $Cd^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ were studied at different membrane potentials. We observed that the efficiency of the block by these divalent cations depended upon the voltage during a step. The voltage-dependent block by $Cd^{2+}$ is illustrated in Fig. 7*A*. The lower inset shows currents recorded during a step to $-28$ mV in the absence and in the presence (dotted line) of 2 $\mu$M $Cd^{2+}$. The upper inset shows the corresponding currents during a step to $+12$ mV. Clearly, the block by 2 $\mu$M $Cd^{2+}$ is much less efficient at $+12$ mV (about 15% at 6 s) than at $-28$ mV (about 85%). The varying efficiency of the block can be appreciated on the graph in Fig. 7*A* which represents the normalized proton conductances remaining in the presence of various $Cd^{2+}$ concentrations at three different voltages $-28$ (□), $-8$ (△)

and +12 mV (◇). Michaelis–Menten equations with different $K_m$ values were an adequate fit to the three sets of conductances. It can be seen in Fig. 7*Ba* that the values of the $K_m$ for $Cd^{2+}$ increased as an exponential function of the voltage. Similar results were obtained with $Ca^{2+}$ (Fig. 7*Bb*) and $Mg^{2+}$. Because $Mg^{2+}$ is much less

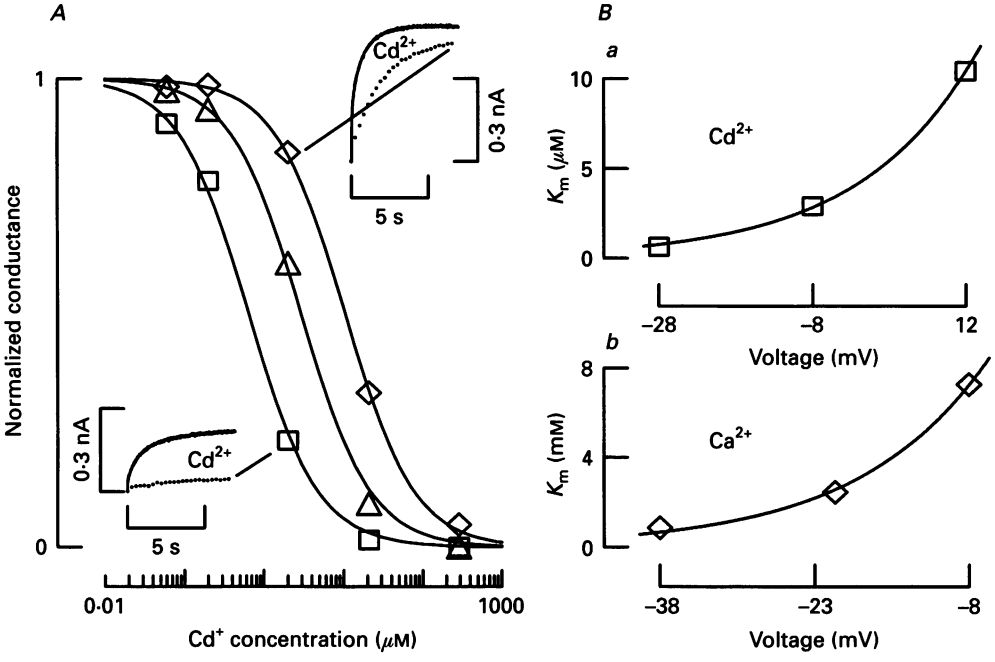


Fig. 7. The block by divalent cations is voltage dependent. *A*, a myotube was held steadily at −68 mV and stepped to either −28 (□), −8 (△) or +12 mV (◇) for 6 s. The intracellular solution was solution C. This voltage step protocol was first applied in a control extracellular solution (solution 2 in which 20 mM aspartic acid was substituted by an equal concentration of HCl) and then in a series of solutions containing free $Cd^{2+}$ at concentrations of 0·06, 0·2, 2, 21 and 287 μM. Solutions containing $Cd^{2+}$ were control solutions (solution 2 with 20 mM $Cl^-$) in which $CdCl_2$ was added and HCl reduced to keep the $Cl^-$ concentration constant. The osmolarity was kept constant using NMG-aspartate and the pH was always 8·00. The lower inset in *A* illustrates current recordings in the absence (continuous line) and in the presence of 2 μM free $Cd^{2+}$ (dotted line) during a step to −28 mV (the current recorded in 287 μM free $Cd^{2+}$ (considered as the 100 % block) has been subtracted). The proton conductance remaining at this voltage in the presence of 2 μM free $Cd^{2+}$ was normalized to the conductance measured in the absence of $Cd^{2+}$ and plotted as a square on the semi-logarithmic graph. The upper inset shows corresponding current recordings obtained at +12 mV. The normalized conductance was plotted as a diamond. Continuous lines on the graph correspond to the equation $g/g_{max} = 1-(1/1+K_m[Cd^{2+}])$. The fit to the data gave $K_m$ of 0·6, 2·9 and 10·4 μM at −28, −8 and +12 mV, respectively. *B*, upper part, plot of the Michaelis–Menten constants for $Cd^{2+}$ as a function of the voltage during a step. The continuous line is an exponential ($y = A\exp(V/B)$) fitted to the data. *B*, lower part, similar results for the block by $Ca^{2+}$. $K_m$ values were 0·9, 2·5 and 7·3 mM at −38, −23 and −8 mV, respectively.

potent than $Cd^{2+}$ or $Ca^{2+}$, only two values of $K_m$ could be determined for this ion (13 and 79 mM at −28 and −8 mV, respectively). Assuming a constant exponential relationship between the $K_m$ for the block by $Ca^{2+}$ and the membrane potential

between −80 and +30 mV, we estimated that 1 mM external $Ca^{2+}$ would reduce $I_H$ by as much as 98% at −80 mV (near resting potential) but by less than 1% at +30 mV (peak voltage during the action potential).

*$I_H$ activation during an action potential*

We wondered whether $I_H$ could be activated during an action potential. Therefore, $I_H$ was recorded at the best possible resolution of our voltage clamp system. In Fig.

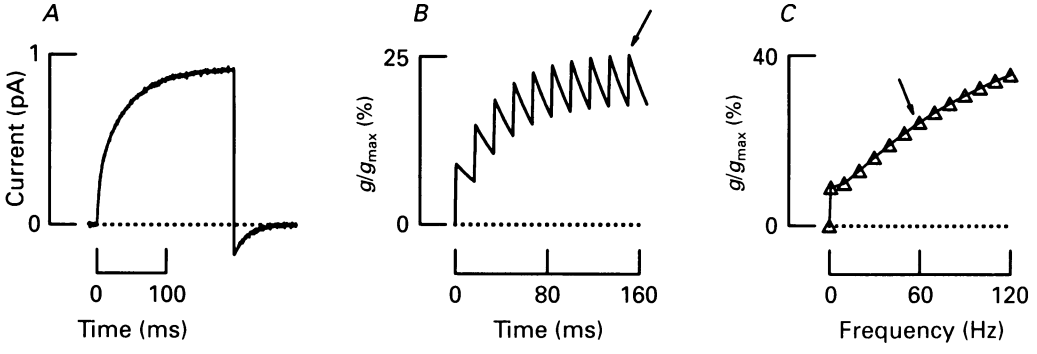


Fig. 8. Activation of $I_H$ during an action potential: a model. *A*, a myotube was depolarized to +42 mV for 200 ms from a holding level of −68 mV and then hyperpolarized to −98 mV. $I_H$ activation at +42 mV could be adequately described with kinetics of the form $(1-\exp(-t/\tau 1))+(1-\exp(-t/\tau 2))$ with $\tau 1 = 4$ ms and $\tau 2 = 44$ ms. The deactivation of $I_H$ at −98 mV had kinetics of the form $\exp(-t/\tau 3)$ with $\tau 3 = 19$ ms. Current is leak-subtracted (the current remaining after application of 5 mM $Cd^{2+}$ in the external medium was considered to be the leak current). The sampling rate was 5 kHz and the low-pass filter was set at 2 kHz. Recording was done in solution 5; intracellular solution was solution A. *B*, $I_H$ build-up (expressed as a percentage of the maximum conductance) during action potentials firing at 60 Hz. Each action potential activates 9% (degree of activation of $I_H$ during the first millisecond of a voltage step to +42 mV; mean of 3 recordings) of the available conductance. The available conductance is defined as the maximum conductance ($g_{max}$) minus the conductance already activated by previous action potentials. The rate constant of deactivation is 46 ms (mean of 4 recordings). *C*, maximum $I_H$ conductance activated during a train of action potentials as a function of the frequency of firing. The arrows in *B* and *C* indicate the maximum conductance reached during a train of action potentials when the firing rate is 60 Hz.

8*A* the current during a step to +42 mV and its decay at −98 mV are illustrated. Resolution in the millisecond range or less requires special recording conditions which we did not apply here. However, our data suggest that a substantial activation of $I_H$ can occur during the first millisecond; in three cells, back extrapolation of current traces suggested that 9±3% of the current was activated during the first millisecond of a depolarizing step. Examination of the deactivation of $I_H$, on the other hand, indicated that the time constant of deactivation at −98 mV was 46±12 ms ($n = 4$).

These values of activation and deactivation were used to compute the progressive build-up of the proton conductance during ten successive action potentials (Fig. 8*B*). It can be seen that the conductance increases progressively up to a saturating level. This final level of activation depends upon the frequency of action potentials, as is

seen in Fig. 8*C*. This figure indicates that at an action potential frequency of 120 Hz, which can be reached at the beginning of a ballistic movement (Desmedt & Godaux, 1977), the conductance of $I_H$ can reach 40 % of its maximum value after less than ten action potentials.

## DISCUSSION

This study reports the existence of a proton current in cultured myotubes obtained from human skeletal muscle. It is the first demonstration of a proton current in a vertebrate excitable cell; previous studies on proton currents have been done with invertebrate neurones or with non-excitable cells. We find that $I_H$ activation depends upon membrane potential and extracellular pH, that this proton transport mechanism is highly selective for protons, and that the elementary conductance of $I_H$ is below the resolution of available techniques. We cannot completely exclude the possibility that the current we studied is due to protons moving through an as yet undescribed type of voltage-gated $K^+$ channel with very unusual characteristics. In addition, we find that several divalent cations block $I_H$, and that the block by $Ca^{2+}$, $Cd^{2+}$ and $Mg^{2+}$ is voltage dependent, being most efficient at hyperpolarized voltages. As a consequence, extracellular calcium at physiological concentration should block $I_H$ at hyperpolarized voltages but not when the membrane is depolarized during action potentials. We suggest that $I_H$ may play a role in proton extrusion during exercise by allowing protons to move out of the muscle cells during action potentials.

Due to the combination of (i) the lack of a selective blocker for $I_H$, (ii) the blocking effect of extracellular $Ca^{2+}$ at hyperpolarized voltages, (iii) the presence of large voltage- and $Ca^{2+}$-gated potassium currents, and (iv) the existence of changes with time and pH of some of these 'parasite currents', we have not yet been able to demonstrate the existence of $I_H$ under physiological ionic conditions on both sides of the membrane.

### *Properties of $I_H$ in human myotubes and other preparations*

The properties of $I_H$ in muscle resemble those of the proton currents described in invertebrate neurones (Byerly *et al.* 1984), axolotl oocytes (Barish & Baud, 1984), rat alveolar epithelial cells (DeCoursey, 1991), or granulocytes (Demaurex *et al.* 1993), although the activation of $I_H$ in the two latter preparations is slower. Like in neurones, $I_H$ of human myotubes activates rapidly at depolarized potentials (see Fig. 8*A*), a quality which may have evolved as an adaptation to the excitable properties of these cells.

As in all preparations studied so far, $I_H$ in myotubes does not require intracellular supply of ATP, nor does it show any run-down over long periods of time. The threshold of activation of $I_H$ and, more generally, the conductance–voltage relationship moved towards positive voltages when the extracellular pH was reduced. Although we did not change the intracellular pH within the course of an experiment, the currents recorded during experiments done at an intracellular pH near 7·3 had a much slower time course than the corresponding currents recorded at more acidic pH. This would be consistent with a shift toward positive voltages of the conductance–voltage relationship when the intracellular pH becomes more basic, as was described in snail neurones (Byerly *et al.* 1984) and in granulocytes (Demaurex *et al.* 1993). Taken together, the results on pH changes on either side of the membrane

suggest that a reduction of the transmembrane pH gradient (rather than the absolute proton concentrations) tends to shift the conductance–voltage relationship towards more depolarized voltages. As suggested by others (Barish & Baud, 1984; DeCoursey, 1991) the current–voltage properties of $I_H$ seem to behave in such a way that the movement of protons is exclusively outward at any transmembrane proton gradient (with the exception of inward relaxation (tail) currents upon returning from a voltage at which $I_H$ was activated).

As $I_H$ is electrogenic, electroneutrality has to be restored inside the cell. In voltage clamp experiments the counter-ion is provided by the voltage clamp system. During physiological functioning, part of the ions that move during an action potential could contribute to electroneutrality. Lactate might be another candidate. Whether the lactate transporter co-transports lactate and protons or transports only lactate is still controversial (Sahlin, Harris, Nylind & Hultman, 1976; Benadé & Heisler, 1978; Chirtel, Barbee & Stainsby, 1984; Roth & Brooks, 1990). Cinnamate did not affect $I_H$ in our voltage clamp experiments (data not shown), but this evidently does not exclude some collaborative interactions between the two transporters under physiological conditions.

*$I_H$ and divalent cations*

The efficiency of the block by divalent cations of the proton current in muscle and snail neurones is similar. An important difference, however, between the two preparations concerns the effect of $Ca^{2+}$: in snail neurones, $Ca^{2+}$ did not affect $I_H$ whereas it had a blocking effect in cultured myotubes. The block by $Ca^{2+}$ followed Michaelis–Menten kinetics and the $K_m$ increased exponentially with membrane potential in the voltage range studied. This suggests that a physiological concentration of $Ca^{2+}$ might block $I_H$ to various extents depending upon membrane potential. The possible physiological implications of this mechanism will be examined below. Voltage-dependent interactions between $Cd^{2+}$ and $Ca^{2+}$ channels have been described in heart muscle, but in this case hyperpolarization rather than depolarization relieved the block (Lansman, Hess & Tsien, 1986).

The block by $Zn^{2+}$ in snail neurones was also reported to follow Michaelis–Menten kinetics (Mahaut-Smith, 1989), suggesting a receptor-mediated effect. In myotubes, a similar mechanism describes the block by $Cd^{2+}$, $Ca^{2+}$ and $Mg^{2+}$. The voltage-dependent increase in $K_m$ reported here for the block by $Cd^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ may explain the shift towards positive potentials of the current–voltage relationship that has been reported in all preparations in which the effect of $Cd^{2+}$ or $Zn^{2+}$ on proton current was studied (Byerly *et al.* 1984; Barish & Baud, 1984; DeCoursey, 1991). In myotubes the time course of $I_H$ changes in the presence of $Cd^{2+}$. This was also reported in other preparations and was interpreted as the result of a direct action of this cation on the current-generating mechanism (Byerly *et al.* 1984). Another explanation of the change in time course could be a time-dependent release of the $Cd^{2+}$ block due to a time-dependent change of $K_m$ during a depolarizing step.

*What could be the physiological role of $I_H$ in muscle?*

In general, and the present study is no exception, $I_H$ has been studied under non-physiological conditions; superfusion solutions and intracellular solutions contained various non-permeant ions to eliminate voltage-dependent currents other

than $I_H$, and the intra- and extracellular pH was set to extreme values in order to increase the amplitude of the current. It should be noted, however, that the intracellular pH we used was not unreasonably low, as a pH of 6·3 was measured with $^{31}P$ nuclear magnetic resonance spectroscopy during exhaustive anaerobic forearm exercise in humans (Hood *et al.* 1988). In addition, a proton current could be recorded at intracellular pH around 7·3 (see Fig. 4), which is probably more basic than the pH of resting muscle (Molé *et al.* 1985; Hood *et al.* 1988). Thus, $I_H$ is probably not an experimental artifact and it is likely that it can be activated under physiological conditions.

A possible way for $I_H$ to operate would be by accumulation of protons near the intracellular side of the membrane. A local decrease of pH could shift the current–voltage characteristics of $I_H$ to hyperpolarized voltages in such a way that protons are expelled even at resting potential. However, such a proton extrusion mechanism could work only if the $K_m$ for the block by $Ca^{2+}$ decreased with a drop of the intracellular pH in the vicinity of the membrane, or else if the outward movement of protons were able to physically displace the $Ca^{2+}$ ions blocking the channels. We could test neither of these two possibilities.

Another proposal, possibly more attractive, for the functioning of $I_H$ is that it operates when the muscle is firing action potentials. During exercise, the firing rate of muscles can oscillate between 30 and 120 Hz (Desmedt & Godaux, 1977), the highest frequency occurring transiently during ballistic movements. Could it be that $I_H$ extrudes a significant amount of protons under such conditions?

An evident requirement for such a mechanism to play a role is that $I_H$ must be minimally activated during a single action potential. Our results suggest that 9% of the maximum conductance may already be activated during the first millisecond of a voltage step to +42 mV. If the current is indeed activated during an action potential, an additional increase in conductance could occur at each spike if the relaxation current of $I_H$ did not decay to zero during the interspike interval. In such a way, there would be a progressive build-up of the proton conductance during a train of spikes up to a saturating value. Figure 8*B* and *C* indicates that such a process could indeed take place in muscle, and that the proton conductance might reach up to 40% of its maximum value, in the best possible conditions.

A problem with this mechanism is that it could also lead to an important proton flux into the cytoplasm during the interspike interval, when the membrane potential is near −80 mV (note that the reversal potential of $I_H$ is somewhere between −17 mV (resting conditions) and −70 mV (acidic conditions)). The voltage-dependent block by extracellular $Ca^{2+}$ may find a physiological role here: $Ca^{2+}$ may prevent an entry of protons via $I_H$ when $I_H$ relaxes after an action potential. When the cell begins to fire a new spike and if there remains a residual proton conductance, protons move outward again as soon as the calcium block is relieved. Of course, the time required for $Ca^{2+}$ to leave its blocking site could be a serious rate-limiting step and will require further study. The effect of $Ca^{2+}$ at voltages more depolarized than −10 mV was difficult to evaluate due to the interference of voltage-dependent $Ca^{2+}$ currents.

What is the proton extrusion power of $I_H$? The current illustrated in Fig. 1*A* at +30 mV corresponds to an $H^+$ efflux of 1·1 mequiv $min^{-1}$ (l tissue)$^{-1}$. Thus, the

proton extrusion power of $I_H$ may seem relatively important, but it must be recalled that during heavy exercise lactic acid is produced at a high rate (Juel, Bangsbo, Graham & Saltin, 1990) and that the proton current may be activated only during action potentials. Thus, at the highest possible frequency of action potentials $I_H$ might extrude no more than 2% of the acid charge produced. It is possible, however, that $I_H$ has a better performance *in vivo*, due to the higher temperature of the human body (our experiments were done at room temperature), or due to the presence of intracellular messengers or extracellular hormones during heavy exercise.

Another possible role for $I_H$ may be to create transiently, during an action potential, a relative proton sink below the membrane during intracellular acidosis. This could generate a pulsing driving force facilitating proton diffusion from the core of the muscle fibre towards the membrane, and may allow membrane proteins to perform at a pH higher than the average intracellular pH. Many membrane channels are known to be sensitive to intracellular pH. In this context it is worth recalling that a low intracellular pH markedly reduces the inactivation of the sodium current in frog skeletal muscle and apparently also slows its activation (Nonner, Spalding & Hille, 1980). The latter process could explain the lower velocity of propagation of the action potential observed in rat muscle during intracellular acidosis (Juel, 1988). Reduced rate of activation and inactivation might also explain the increase by 30 to 60% of the duration of the action potential at a calculated intracellular pH of 6·4 (Juel, 1988). ATP-dependent and $Ca^{2+}$-dependent potassium channels were also found to be affected by intracellular pH (Laurido, Candia, Wolf & Latorre, 1991; Davies, Standen & Stanfield, 1992). Thus, if $I_H$ can indeed create a less acidic zone underneath the membrane, perturbed ionic channel functioning at acidic pH values might be delayed.

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