Transmembrane Outward Hydrogen Current in Intracellularly Perfused Neurones of the Snail *Helix Pomatia*

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Abstract. The ionic nature and pharmacological properties of the outward current activated by membrane depolarization were studied on isolated neurones of the snail *Helix pomatia*, placed in Na^+- and Ca^{2+}-free extracellular solutions and intracellularly perfused with K^+-free solution ("nonspecific outward current"). It was shown that the amplitude and reversal potential of this current (estimated from instantaneous current-voltage characteristics) are determined mainly by the transmembrane gradient for H^+ ions. Lowering of pH_i induced an increase in the current amplitude and a shift of the reversal potential to more negative values; the shift magnitude was comparable with that predicted for the hydrogen electrode. Raising pH_i, as well as lowering pH_o, induced a decrease in the current amplitude and a displacement of the current activation curve to more positive potentials. Addition of EGTA (8 mmol/l) to the intracellular perfusate did not affect the current amplitude. Extracellular 4-aminopyridine (10 mmol/l), verapamil (0.25 mmol/l) or Cd^{2+} (0.5 mmol/l) blocked the current. It is concluded that the current studied is carried mainly by H^+ ions.

In the same neurones the nature of the fast decay of the calcium inward current was also studied (in the presence of extracellular Ca^{2+} ions). This decay considerably slowed when pH_i was raised or pH_o was lowered, and it became less pronounced upon extracellular application of 4-aminopyridine or upon intracellular introduction of phenobarbital (4 mmol/l) and tolbutamide (3 mmol/l). It is suggested that the fast decay of the calcium inward current is due to activation of a Ca-sensitive component of the hydrogen current which depends on accumulation of Ca^{2+} ions.

The possible physiological role of the transmembrane hydrogen currents is discussed.

Key words: Hydrogen ions — Potential-dependent outward current — Helix neurones

Introduction

Beginning from the pioneering work by Kostyuk and Krishtal (1977) the non-specific outward current described by these authors and later by Byerly and

Hagiwara (1982) in the membrane of intracellularly perfused snail nerve cells was regarded as an artifact of a non-ideal selectivity of ionic channels. Recent experiments have shown that the current could be significantly decreased by raising the pH value of the intracellular perfusate (Doroshenko and Martynyuk 1982; Doroshenko et al. 1984). These data as well as those concerning a depolarization-induced increase in the permeability to H^+ ions of the membrane of nonisolated, nonperfused nerve cells (Thomas and Meech 1982) led us to propose that the nonspecific outward current could be carried by H^+ ions (Doroshenko et al. 1984). The outward current carried by H^+ ions was also implicated in the development of the fast phase of the Ca inward current decay during sustained depolarizations (Doroshenko et al. 1982, 1984). The present paper contains results of the experimental testing of these suggestions. The results have already been presented at the 2nd International Symposium on Ionic Channels and Membrane Receptors (21—26 May 1984, Smolenice, Czechoslovakia) and published in part elsewhere (Doroshenko and Martynyuk 1985). The results obtained and conclusions made partly coincide with those presented recently by Byerly et al. (1984).

Materials and Methods

Experiments were performed on nonidentified isolated nerve cells of the snail *Helix pomatia*. The techniques of cell isolation after enzymatic treatment (Pronase E, Serva, 0.2 %, 2 h at room temperature), intracellular perfusion and voltage clamp were similar to those described previously (Kostyuk et al. 1981). Electronic compensation was used to eliminate the series resistance effects on the voltage clamp fidelity. In majority of experiments the transmembrane ionic currents were recorded after summing the currents elicited by successive depolarizing and hyperpolarizing pulses of the same amplitude and duration. This procedure ensures elimination of capacitative and leakage currents linearly changing with the membrane potential. In some experiments, when very large depolarizing pulses were necessary or the membrane leakage became increased due to the experimental influences, an analog compensation for the leakage conductance measured by applying small ($-20 \div -25$ mV) hyperpolarizing pulses from the holding potential (usually -50 mV) was used instead of current summation.

The extracellular solutions used contained (in mmol/l): 20 $CaCl_2$; 4 $MgCl_2$; 95 Tris-Cl; pH 7.5. In Ca-free solutions $CaCl_2$ was replaced by $MgCl_2$. Additions of other substances to these solutions were performed by replacing equivalent amounts of Tris-Cl. The basic intracellular perfusate contained (in mmol/l): 127 Tris-aspartate; 3 theophylline; pH 7.3. To raise or to lower the pH value of the perfusate, or to change free calcium level in it, definite amounts of Tris-aspartate were replaced by Tris-OH, MES (usually 30 mmol/l) or EGTA (8 mmol/l).

The substances used were: Tris (Tris-(hydroxymethyl)aminomethane, Serva); MES (Morpholinoethane sulfonic acid, Serva); EGTA (Ethylene glycol bis(2-aminoethyl ether)-N,N'-tetraacetic acid, Serva). All experiments were carried out at room temperature (18—21 °C).

Results

Ca-independent currents. In the first series of experiments the transmembrane currents were studied in the absence of sodium, potassium and calcium ionic

gradients, respectively. To eliminate the latter, extracellular calcium was replaced by magnesium; the former two, extracellular and intracellular Na^+ and K^+ ions, were replaced by Tris ions. Despite the absence of the principal current-carrying ions, in all the cells studied depolarization of the membrane activated time- and potential-dependent outward current. When pH values of the extracellular (pH_o) and intracellular (pH_i) solutions were buffered at their corresponding physiological values (pH_o 7.5 and pH_i 7.3), this current appeared at depolarizations exceeding zero membrane potential level. Examples of the current records and their current-voltage characteristics are shown in Fig. 1. Upon membrane depolarization the current increased rapidly to a stationary level depending on the amplitude of the depolarizing pulse. It did not inactivate during prolonged (up to several hundreds of milliseconds) depolarizations. The kinetics of the current activation could be approximated by a single exponential with time constant decreasing with depolarization (from 15—20 ms at +10 mV to 3—5 ms at +60 mV). Addition of 8 mmol/l EGTA to the perfusate did not affect the current amplitude. The current remained constant in the amplitude during long periods (more than 1 h) of intracellular perfusion, being obviously independent of the cellular metabolism.

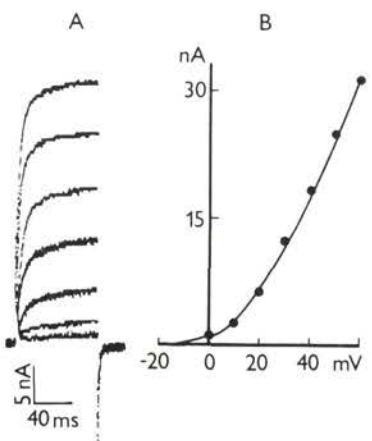

Fig. 1. Current records (*A*) and the corresponding I—V relationship (*B*) for the transmembrane outward current activated by depolarization in a neurone placed in a Ca-free medium and intracellularly perfused with K-free solution. $pH_o/pH_i = 7.5/7.3$. Holding potential −50 mV, test pulses to 0; 10; 20; 30; 40; 50 mV.

Following repolarization of the membrane to the holding potential level (usually −50 mV), current tails of inward direction were recorded, suggesting a more positive value of the current reversal potential as compared with the holding potential. (The Nernst potential for H^+ ions in specified conditions was −11 mV).

The amplitude of the outward current under study definitely depended upon

the transmembrane gradient of H^+ ions. An increase in their intracellular concentration (lowering pH_i) at constant pH_o induced an increase in the current amplitude, a reversal of the current tails direction to outward, and a shift of the current-voltage curve to more negative membrane potentials. This is illustrated in Fig. 2, where two groups of outward current records and the corresponding I—V curves obtained on the same cell at pH_o 7.5 and pH_i 7.3 (the upper group) and 5.8 (the lower group) are shown. The Nernst potential for hydrogen ions at the lower pH_i was −98 mV. It should be noted that a relative increase in the current amplitude, though considerable, was less than that predicted from the increase in the H^+ ion concentration.

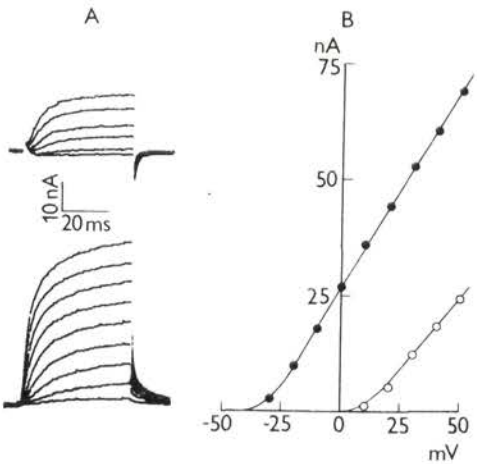

Fig. 2. Changes in the outward current induced by lowering pH_i. *A* — current records obtained under conditions of physiological $pH_o/pH_i = 7.5/7.3$ (upper set) and after lowering pH_i to 5.8 (lower set) at the same pH_o. Holding potential −50 mV, test pulses to 0; 10; 20; 30; 40; 50 mV for pH_i 7.3, and −30; −20; −10; 0; 10; 20; 30; 40; 50 mV for pH_i 5.8. *B* — I—V relationships for the outward current constructed from the current records shown in *A*: open circles — pH_i 7.3, closed circles — pH_i 5.8.

The current increase was also accompanied by an increase in the slope of the I—V curve which reflected an increased conductance of the membrane. As expected, raising pH_i (i.e. lowering the intracellular H^+ ion concentration) at constant pH_o induced in all cases a decrease in the current amplitude. Fig. 3 shows the corresponding I—V curves for the current at two values of pH_i (7.3 and 8.2). In addition to the above changes in the current amplitude, raising pH_i also produced a considerable shift of the I—V curve to more positive potential values and a decrease in its slope. The current activation was observed only at very high depolarizations exceeding +40 mV (which coincided with the Nernst potential for H^+ ions under such conditions).

In some experiments, an outward current was recorded after raising pH_i at the

same depolarizations as at initial pH_i (Fig. 3*B*). However, its time course (similar to that of the voltage pulse), the absence of tail currents at the end of the corresponding depolarizations, and a linear dependence on the voltage allow us to suggest that it originated from the increased rectification in conditions of high pH_i. After subtraction of this current, the remaining one showed usual voltage-dependence with the I—V curve displaced to large depolarizations.

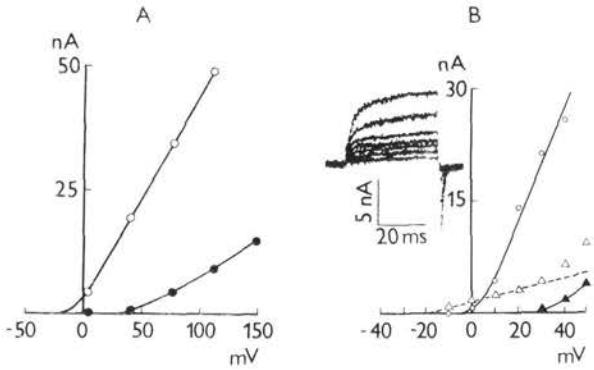

Fig. 3. Changes in the outward current induced by raising pH_i. *A* — I—V relationship for the current obtained under conditions of $pH_o/pH_i = 7.5/7.3$ (open circles) and after changing pH_i to 8.2 at the same pH_o (closed circles). *B* — another example of the effect of high pH_i. Open circles mark the I—V curve for the standard conditions of $pH_o/pH_i = 7.5/7.3$, triangles — after a change in pH_i from 7.3 to 8.2. Changes in the current amplitude were accompanied by an increase in leakage. Results of subtraction of presumed leakage currents from the total current are shown by closed triangles (see text). Inset shows original current records obtained after raising pH_i. Holding potential −50 mV, test pulses to −10; 0; 10; 20; 30; 40; 50 mV. Note that time-dependent currents and current tails appeared only at depolarizations exceeding 30 mV.

It is important to note that despite the presence of inward tail currents, hydrogen currents of inward direction were not observed in any case.

The effects on the outward current of changes in transmembrane pH gradient produced by lowering pH_o at fixed pH_i (7.3) were also studied. In Fig. 4 the results of pH_o lowering from 7.5 to 6.6 are shown. The current tails became considerably enlarged, the I—V curve was displaced to the right so that the outward current was activated only at depolarizations above +40 mV (similarly as in the case of high pH_i, Fig. 3, this corresponded to the Nernst potential for H^+ ions in the new conditions). Smaller depolarizations elicited neither outward nor inward currents. Current tails were also absent. The I—V curve for the outward current was shifted almost parallelly to the right without noticeable change in its slope.

The selectivity of the outward current channels was studied by measuring the reversal potential (E_{rev}) at two different values of pH_i. Fig. 5 shows the instantane-

ous I—V relationships for the outward current obtained at two pH_i values (7.3 and 5.9) and a constant pH_o (7.5). The records show changes in the tail currents following repolarization to different levels of the membrane potential after a depolarizing 50 ms pulse to +30 mV (from a holding level of −52 mV) (Fig. 5*A* at pH_i 7.3 — left, at pH_i 5.9 — right). Initial tail current amplitudes measured in about 0.5 ms after the termination of the depolarizing pulse were plotted against the corresponding repolarization levels of the membrane potential (Fig. 5*B*). The instantaneous I—V relationships could be satisfactorily approximated by straight lines in both cases. At physiological values of pH_o and pH_i the measured reversal potential for the outward current (−10 ±3 mV) (mean of 4 cells) appeared to be rather close to the Nernst potential for H^+ ions (−11 mV). After lowering pH_i to 5.9 the measured value of E_{rev} was −64 ±2 mV (mean of 4 cells). The average shift of the reversal potential was −54 mV. The hydrogen electrode potential in these conditions should change by −81 mV. In one experiment the measured shift of E_{rev} induced by lowering pH_i from 7.4 to 5.9 was −92 mV (the theoretical value −87 mV).

The saline solutions bathing the cell membrane also contained $Tris^+$, Cl^-, and aspartate $^-$ions. The latter anions do not penetrate through the membrane and

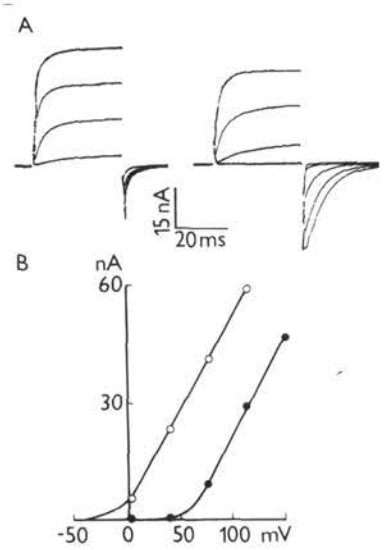

Fig. 4. Changes in the outward current induced by lowering pH_o. *A* — two sets of current records obtained for identical test pulses to 4; 40; 76; 112; 148 mV (from a holding level of −50 mV) in the same cell at pH_o 7.5 (*left*) and pH_o 6.6 (*right*). pH_i was kept constant at 7.3. Note that the currents elicited by depolarizations to 4 and 40 mV in the right set nearly coincided. The current tail following depolarization to 148 mV is off-scalle. *B* — I—V relationships constructed from the currents shown in *A*: pH_o 7.5 — open circles, pH_o 6.6 — closed circles.

could not create outward current at membrane depolarization. Changes in the extracellular Cl^- ion concentration did not practically affect the outward current amplitude. Lowering of the intracellular concentration of $Tris^+$ ions from 130 to 30 mmol/l (equimolar replacement for imidazole) induced a small decrease (to about 70 % of the original value) in the outward current amplitude.

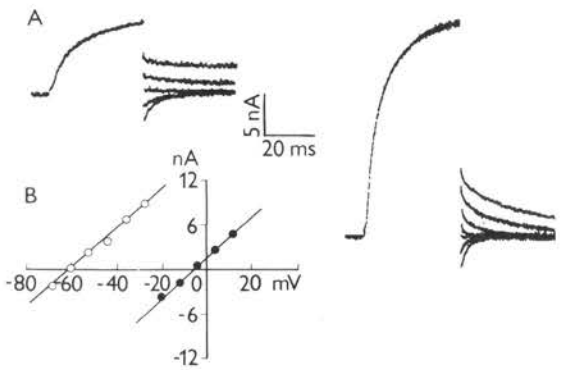

Fig. 5. Instantaneous I—V relationships for the outward current measured for two transmembrane pH gradients. *A* — the outward current (depolarization to 30 mV from a holding level of −52 mV) and the current tails following repolarizations to −20; −12; −4; 4; 12 mV at $pH_o/pH_i = 7.5/7.3$ (*left*), and to −84; −68; −60; −52; −44; −36; −28 mV at $pH_o/pH_i = 7.5/5.9$ (*right*). *B* — the instantaneous I—V relationships constructed from the current records shown in *A*: $pH_o/pH_i = 7.5/7.3$ — closed circles, $pH_o/pH_i = 7.5/5.9$ — open circles.

Pharmacological properties of channels carrying the outward current described were also studied. The current was reversibly blocked by extracellular application of a K^+-channel blocker, 4-aminopyridine (4-AP, Hermann and Gorman 1981) and Ca^{2+}-channel blockers, verapamil and Cd^{2+} ions (Kostyuk and Krishtal 1977). Fig. 6 illustrates the blocking action of 10 mmol/l 4-AP (Fig. 6*A*) and 0.5 mmol/l Cd^{2+} ions (Fig. 6*B*) on the outward current. Tetraethylammonium (20 mmol/l) or quinine (1 mmol/l, Hermann and Gorman 1984), other known blockers of K^+-channels, had a much lesser inhibitory effect on this current.

Ca-sensitive current. A second series of experiments was carried out in the presence of Ca^{2+} ions in the extracellular medium. Under these conditions, membrane depolarization produced a calcium inward current which decayed (inactivated) during sustained depolarization. It is known that the current decay consists of two, fast and slow, components (Kostyuk and Krishtal 1977; Doroshenko et al. 1984). Both of them may be due to accumulation of Ca^{2+} ions at the inner surface of the membrane (Plant and Standen 1981; Eckert and Chad 1984). However, it still remains unresolved whether the fast decay of the Ca inward current is a result of actual inactivation (i.e. decrease) of calcium conductance, or

whether it is due to superposition upon the Ca current of an outward current carried by other ions and activated by Ca^{2+} ions entering the cell. Evidence for the latter suggestion has been given by us in a preceding paper (Doroshenko et al. 1984).

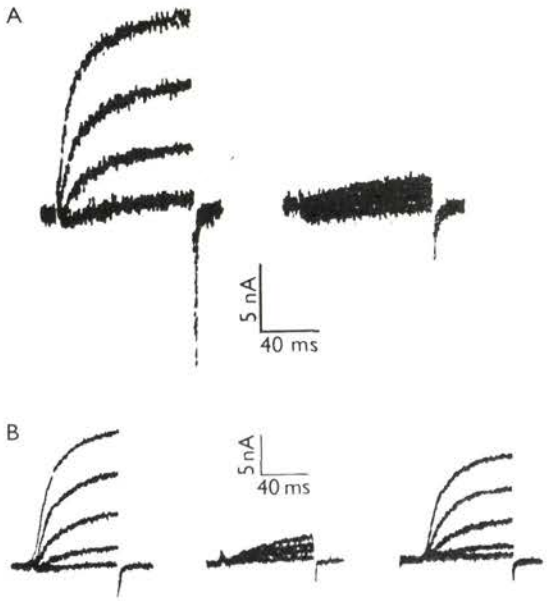

Fig. 6. Blockade of the outward current. *A* — current records obtained before (*left*) and after (*right*) the addition of 10 mmol/l 4-AP to the extracellular solution. Holding potential −50 mV, the same test pulses to 10; 20; 30; 40 mV. $pH_o/pH_i = 7.5/7.3$. *B* — current records obtained before (*left*), about 1 min after the addition of 0.5 mmol/l Cd^{2+} ions to the extracellular solution (*middle*) and 10 min after their removal (*right*). Holding potential −50 mV, test pulses to 0; 10; 20; 30; 40 mV. $pH_o/pH_i = 7.5/7.3$.

If the existence of a hydrogen current is assumed, the activation of which also depends on Ca^{2+} ion accumulation inside the cell, the most important of the known experimental findings, namely, Ca-dependence and H-dependence of the fast decay of Ca current can be explained. Following results support the above suggestion. The fast decay of the Ca current was suppressed by the same experimental influences which were shown above to inhibit the outward current of H^+ ions. In Fig. 7*A*, the effect of raising pH_i from 7.3 (upper curve) to 8.2 (lower curve) on the Ca current decay is shown. A similar decrease in the rate of the current decay was also caused by lowering pH_o (also see Doroshenko et al. 1978). Extracellular 4-AP in a concentration as used to block the outward current of H^+ ions (10 mmol/l) also slowed the Ca current decrease during depolarizing pulses (Fig. 7*B*).

Definite effects on the Ca current decay were also produced by intracellular administration of putative inhibitors of protein phosphorylation phenobarbital (Fig. 8*A*, 4 mmol/l) and tolbutamide (Fig. 8*B*, 3 mmol/l). Their action developed in about 10 min; during this time the rate of the Ca current decay gradually slowed down. In most experiments these agents (in the concentrations as above) produced little effect on the amplitude of the above described Ca-independent outward current of H^+ ions.

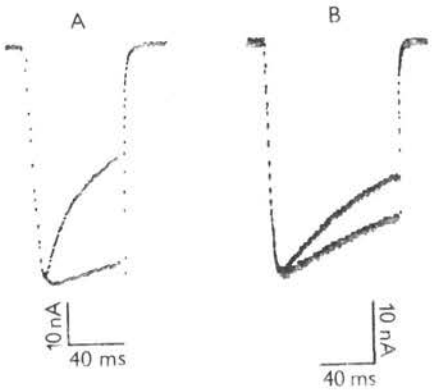

Fig. 7. Changes in the kinetics of the Ca current decline induced by procedures shown to inhibit the outward current. *A* — superposition of two Ca current records (depolarizations to +30 mV from a holding potential level of −50 mV) obtained at pH_i 7.3 (*upper curve*) and pH_i 8.2 (*lower curve*) at constant pH_o 7.5. *B* — superposition of two Ca current records (holding potential −50 mV, depolarizations to +20 mV) obtained before (*upper curve*) and after (*lower curve*) the addition of 10 mmol/l 4-AP to the extracellular solution at constant pH_o 7.5, pH_i 7.3.

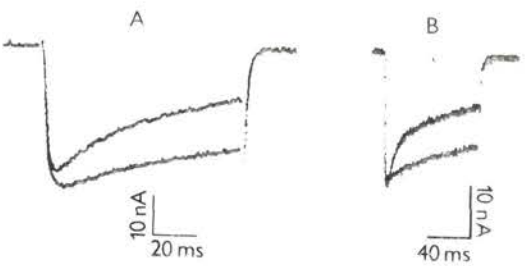

Fig. 8. Effects of intracellular introduction of phenobarbital and tolbutamide on the Ca current decline. *A* — two Ca current records obtained in standard conditions (upper curve) and 12 min after the addition to the perfusate of 4 mmol/l phenobarbital (*lower curve*) are superimposed. *B* — the two Ca current records were obtained in standard conditions (*upper curve*) and 8 min after the intracellular introduction of 3 mmol/l tolbutamide (*lower curve*). Holding potential −50 mV, depolarization to 20 mV, pH_o 7.5, pH_i 7.3.

Discussion

The results described show that the "nonspecific" outward current (or its main portion) recorded in the membranes of intracellularly perfused snail neurones is carried by H^+ ions. This conclusion is supported by the following experimental findings. The amplitude of the outward current was determined by the electrochemical gradient for H^+ ions and changed following changes in either intracellular or extracellular pH. Measurements of the current reversal potential by means of instantaneous current-voltage relationships showed that at physiological values of pH_o (7.5) and pH_i (7.3) it practically coincided with the Nernst potential for H^+ ions, and it shifted to more negative values of the membrane potential when pH_i was lowered. The magnitude of the shift was usually smaller than the expected change in the hydrogen electrode potential that may be explained by insufficient effectiveness of the pH_i control in specified conditions of intracellular perfusion. This is also consistent with the finding that the observed degree of the outward current increase resulting from the perfusion of the cell with solution of low pH_i was smaller than expected.

Another reason may be the participation of other ions together with H^+ ions in producing the outward current. Chloride and aspartate anions can be excluded as discussed above. The contribution of $Tris^+$ ions, however, cannot be excluded at present. The experimental data indicate that the contribution of these latter ions must be relatively small since raising pH_i as well as lowering pH_o produced predominant decreases in the outward current amplitude, while a decrease in the intracellular $Tris^+$ ion concentration induced an only insignificant decrease in the current amplitude. Variations in the Nernst potential for $Tris^+$ ions due to different concentrations of ionized Tris molecules at low and high pH values (at high pH 8.2, about half of them are present in the neutral form) must be too small to produce the changes observed in the current amplitude and reversal potential. Therefore, as in the case of Limnaea neurones (Byerly et al. 1984) the best confirmed conclusion is that the described outward current is carried mainly by H^+ ions through rather selective voltage-dependent ionic channels.

In their pharmacological properties, the channels producing the outward current of H^+ ions in the membrane of Helix neurones are similar to H^+ channels in the Limnaea neurone membrane (Byerly et al. 1984): they are blocked by extracellular application of 4-AP, verapamil and Cd^{2+} ions and are relatively insensitive to the action of TEA and quinine. Their functioning seems to be independent of the concentration of free Ca^{2+} ions in the intracellular perfusate as the addition to the latter of EGTA did not affect the current amplitude (it is prematurely to draw a similar conclusion about the current dependence on the intracellular Ca^{2+} ion concentration because it may not follow the concentration of these ions in the perfusate, see Byerly and Moody 1984).

A characteristic feature of the transmembrane currents carried by hydrogen ions is that even at reversed transmembrane gradient for these ions it was impossible to reverse the direction of the currents (though the ability of the corresponding channels to pass inward flow of H^+ ions was proved by the presence of inward tail currents which increased substantially upon lowering pH_o, and also by practically linear instantaneous current-voltage relationships). This fact can be explained on the basis of large and somehow coordinated shifts of the activation curve for hydrogen current which accompany the changes in either pH_o or pH_i. For example, upon lowering pH_o from 7.5 to 6.6 (in this case at pH_i 7.3, the Nernst potential for H^+ ions changes from -11 to $+40$ mV) observable outward current appeared only at depolarizations exceeding $+40$ mV (Fig. 4). The inward tail currents also appeared in this membrane potential region indicating that the channels were not activated at lower depolarizations. A similar situation was observed during raising pH_i. This feature of the hydrogen current was also reported by Barish and Baud (1984).

The fact that H^+ions can create measurable transmembrane current at normal pH_o and pH_i values may have several interesting consequences. One of them concerns Ca current inactivation. The inevitable presence of the outward hydrogen current during Ca current recording should prompt us to consider the possible participation of the former in the formation of the usually observed Ca current decay during sustained depolarization. Obviously, the above hydrogen current activates faster and is rather small in amplitude (at a given depolarization) to account for the fast phase of the decay, or to considerably affect the overall time course of the Ca current (this argument was put forward earlier by Eckert and Chad 1984). Its activation would rather result in a diminution of the Ca current amplitude. Furthermore, this current seems not to depend on Ca^{2+} ion accumulation.

Nevertheless, raising pH_i (Fig. 7*A*) as well as lowering pH_o (Doroshenko et al. 1978) did slow the Ca current decay, the former being able to completely eliminate the fast phase of the decay (Doroshenko et al. 1984). This difficulty can be overcome, on the assumption that, in the presence of the Ca inward current, an additional component of the outward current is activated, carried by H^+ ions, and its activation depends on Ca^{2+} ion accumulation (Ca-sensitive hydrogen current). The suggested current manifests itself as a fast decay of the Ca current and upon its properties can at present be judged only by the as one indirect findings.

The nature of ionic channels which pass both components of the hydrogen current remain unclear. One possibility is that these channels are a new type of ion selective channels, as suggested by Byerly et al. (1984). The most characteristic feature of these channels must be a very high conductance for H^+ ions which enables them to pass substantial transmembrane currents in conditions of extremely low activity of H^+ ions in physiological solutions. It is also difficult to rule out

that the hydrogen current is produced by already known ionic channels, as recently reported for the nodal sodium channels (Mozhayeva et al. 1982). Kinetic and pharmacological properties of the hydrogen current, especially of its Ca-sensitive component, resemble those of the Ca-dependent K^+ current (Kostyuk et al. 1980). It was shown recently (de Peyer et al. 1982) that the functioning of the Ca-dependent K^+ channel depends on cAMP-dependent phosphorylation. The described effects on the Ca-current decay of intracellularly applied phenobarbital and tolbutamide (agents known to inhibit cAMP-dependent phosphorylation) have confirmed the possibility that the suggested Ca-sensitive hydrogen current also depends on the cyclic nucleotide metabolism.

The Ca-independent hydrogen current may be a result of activity of either the same or a different type of channels independent of cellular metabolic processes. Undoubtedly, further investigations are necessary to explain these anomalous properties of the putative channels producing the hydrogen current.

We believe, in accordance with Byerly et al. (1984) that the described hydrogen current (despite some as yet unexplained experimental findings) is not an artifact of the intracellular perfusion. Rather, the discovery and investigation of this phenomenon have become possible because of the use of the perfusion method. Now it is important to clarify the possible physiological role of the hydrogen current. It may participate in the regulation of pH_i during acidification of the intracellular medium (and depolarization of the membrane) as discussed by Thomas and Meech (1982) and Byerly et al. (1984). Also, it may be suggested that the hydrogen current, especially its Ca-sensitive component, plays a role in the generation of some specific types of electric activities, such as pacemaker membrane potential oscillations.

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Received February 20, 1985/Accepted February 11, 1986