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THE EFFECT OF ZINC ON CALCIUM AND HYDROGEN ION CURRENTS IN INTACT SNAIL NEURONES

BY M. P. MAHAUT-SMITH*

Department of Physiology, Medical School, University Walk, Bristol BS8 1TD

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Summary

The effects of external Zn^{2+} on Ca^{2+} and H^+ currents in the soma of intact *Helix* neurones were investigated using standard two-electrode voltage-clamp procedures. Cells were exposed to a $0Na^+$, tetraethylammonium (TEA^+) saline and clamped with Cs^+-filled electrodes, which allows separation of voltage-dependent H^+ and Ca^{2+} currents using different holding potentials. Outward H^+ currents, activated by depolarizations from holding potentials in the range −15 to −10 mV, were rapidly blocked by low concentrations of external Zn^{2+} with a K_d of approximately 16 μmol l^{-1}. H^+ current activation was also markedly slowed and the block was slow to reverse. Ca^{2+} currents, largely free from contamination by outward current, were activated by small depolarizations from a holding potential of −55 mV. Ca^{2+} currents were reduced by Zn^{2+}, but the K_d for block was more than 80 times greater than for block of H^+ currents. Thus, low concentrations of Zn^{2+} provide a method of selectively inhibiting H^+ current in studies of Ca^{2+} current. This was demonstrated in cells which slowly acidified following exposure to $0Na^+$, TEA^+ saline, leading to an increased outward H^+ current. Washing with low concentrations of Zn^{2+} blocked the H^+ current and uncovered the underlying Ca^{2+} current. The results also suggest that Zn^{2+} will be a useful tool in studies of the physiological role of the H^+ pathway.

Introduction

Voltage-dependent hydrogen ion movements have been detected in a number of tissues (Thomas & Meech, 1982; Byerly *et al.* 1984; Barish & Baud; 1984; Mahaut-Smith, 1987, 1989; Henderson *et al.* 1987; Thomas, 1988*a*,*b*). In molluscan neurones, continuous activation of the H^+ pathway can produce substantial changes in pHi (Thomas & Meech, 1982), but the physiological role of these H^+ movements has not been investigated. A number of agents block H^+ currents, but the most effective are heavy metal cations such as Cd^{2+}, La^{3+} and Zn^{2+}, which also block Ca^{2+} currents in these cells.

This study shows that H^+ currents in intact *Helix* neurones are blocked by micromolar concentrations of Zn^{2+}. Ca^{2+} currents are also blocked by Zn^{2+}, but

* Present address: Physiological Laboratory, Downing Street, Cambridge CB2 3EG.

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at higher concentrations. Zn^{2+} can therefore be used to detect the presence of H^+ currents in studies of Ca^{2+} currents. This blocker may also be useful in studying the role of the H^+ pathway in normal cell function. A preliminary report of the results has appeared in abstract form (Mahaut-Smith, 1987).

Materials and methods

The preparation and experimental set-up were identical to those described in the preceding paper (Mahaut-Smith, 1989). $ZnCl_2$ was obtained from BDH Chemicals.

Results

The previous paper describes conditions which allow the separation of H^+ currents in intact *Helix* neurones. Briefly, cells were held under voltage-clamp using CsCl-filled microelectrodes and exposed to a $0Na^+$, TEA^+ saline. The membrane potential was held at -30 mV for 10 min and finally at -10 or -15 mV. The low holding potentials, and the saline used, induce a cytoplasmic acidification and abolish Ca^{2+} and remaining K^+ currents. As a result, depolarizing pulses from these low holding potentials activate fast outward currents, which are carried by H^+, at least for pulses up to +55 mV (Mahaut-Smith, 1989).

In preliminary experiments, a number of agents known to affect voltage-dependent outward currents or H^+ movements were tested on outward H^+ currents in intact neurones. The following compounds had no significant effect when added to the bath; *N*-ethylmaleimide (2 mmol l^{-1}), diethylpyrocarbonate (1 mmol l^{-1}), amiloride (100 μmol l^{-1}), apamin (Sigma) (0·3 μmol l^{-1}). 1–2 mmol l^{-1} external Cd^{2+} blocked a large proportion of these H^+ currents, but by far the most effective blocker was Zn^{2+}.

Fig. 1A shows H^+ currents activated by a series of depolarizing voltage steps from a holding potential of -15 mV up to 55 mV in 10 mV increments. 0·1 mmol l^{-1} $ZnCl_2$ added to the bath caused a large reduction in the H^+ current, an effect which was slow to reverse and only partially recovered after a 30 min wash in $0Na^+$, TEA^+ saline. Subsequent application of 1 mmol l^{-1} external $ZnCl_2$ virtually abolished the outward current. Hyperpolarizing voltage pulses elicited currents which behaved in an Ohmic fashion. Therefore, leak currents for depolarizing pulses were obtained by extrapolating from the Ohmic relationship, measured regularly throughout the experiment. Fig. 1B is a plot of the peak currents during a 250 ms pulse, corrected for the leak current, as a function of the membrane potential. The reduction in current caused by Zn^{2+} appears to be constant within the range of potentials used, which suggests that the block is not voltage-dependent. In the presence of 1 mmol l^{-1} Zn^{2+}, a small voltage-dependent current was observed during steps to +55 mV or more depolarized, which may be residual K^+ current (Mahaut-Smith, 1989). At these concentrations, divalent cations such as Zn^{2+} and Cd^{2+} have little effect on the voltage-gated K^+ current in

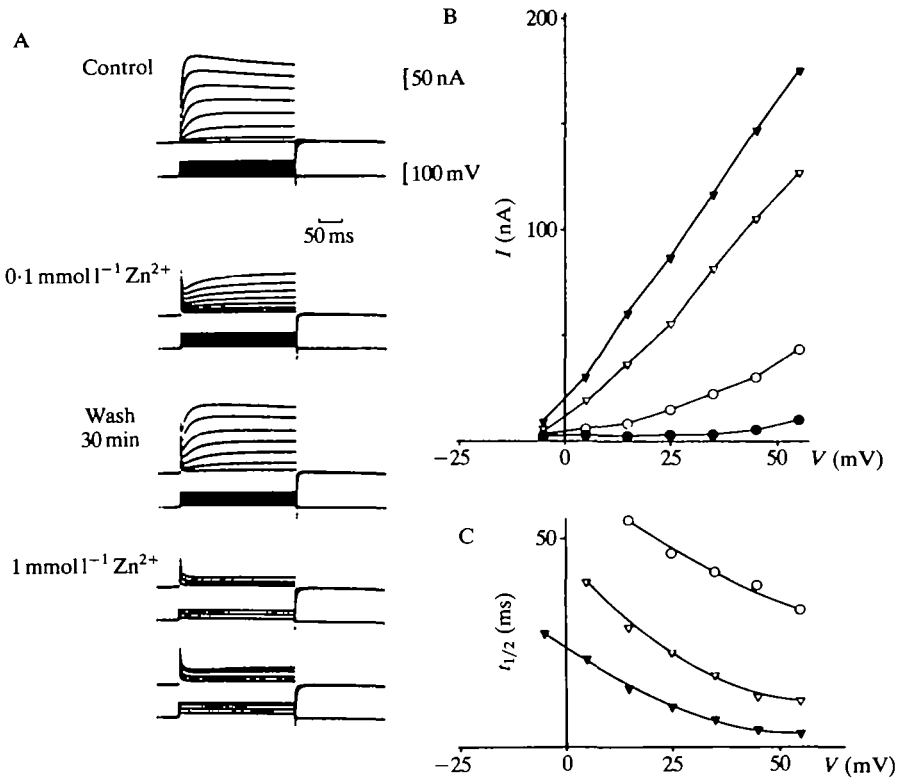

Fig. 1. Effect of Zn^{2+} on H^+ currents. (A) Current (upper) and voltage (lower) records for 250 ms depolarizing voltage steps up to 55 mV in 10 mV increments. Holding potential −15 mV. Order of experiment; control ($0Na^+$, TEA^+); 10 min in 0·1 mmol l^{-1} Zn^{2+}; 30 min wash and 5 min in 1 mmol l^{-1} Zn^{2+}. (B) Leak-corrected current–voltage relationship. Ordinate, peak outward current measured during the 250 ms pulse. Abscissa, membrane voltage. Points are connected by straight lines. (C) Voltage-activation curve. Ordinate, time to half the peak current. Abscissa, membrane voltage. $t_{1/2}$ could not be measured in 1 mmol l^{-1} Zn^{2+} or at lower potentials in 0·1 mmol l^{-1} Zn^{2+} because of the lack of significant outward current. Curves are drawn by eye. ▼, control ($0Na^+$, TEA^+ saline); ○, 10 min in 0·1 mmol l^{-1} Zn^{2+}; ▽, 30 min wash; ●, 5 min in 1 mmol l^{-1} Zn^{2+}.

Helix neurones that are voltage-clamped with KCl-filled electrodes in normal saline (Meech & Standen, 1975; M. P. Mahaut-Smith, unpublished observations).

The rate of activation of the time-dependent current was estimated from the time to half the peak current (see Fig. 1C). Zn^{2+} considerably slowed the activation of the outward current. Note that $t_{1/2}$ could not be measured in 1 mmol l^{-1} Zn^{2+} and at the lower potentials used in 0·1 mmol l^{-1} Zn^{2+} because no significant current was activated.

Kinetics of Zn^{2+} block of H^+ currents

Fig. 2 shows the effect of 0·1 mmol l^{-1} Zn^{2+} on the outward current in response

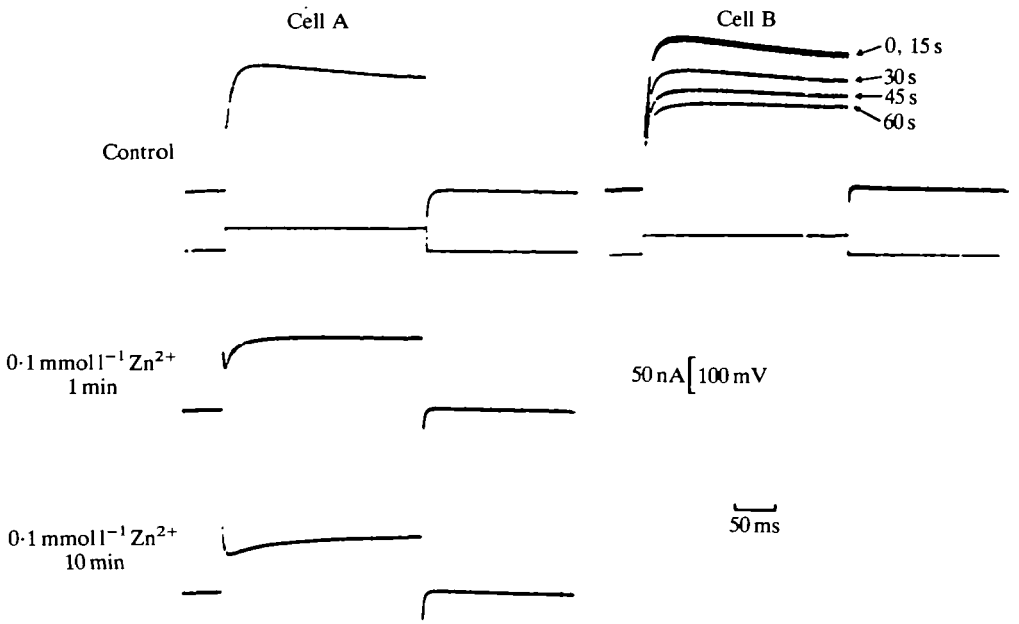

Fig. 2. Time course of block of H^+ currents by 0·1 mmol l^{-1} Zn^{2+}. Cell A, long exposure to Zn^{2+} (10 min). Pulses to 45 mV; holding sequential −15 mV; pulse length 250 ms. Records of membrane current are shown in the control ($0Na^+$, TEA^+) saline, and after 1 and 10 min in 0·1 mmol l^{-1} Zn^{2+}. Cell B, short exposure (1 min) with pulses every 15 s. Pulses to 35 mV; holding potential −15 mV; pulse length 250 ms.

to depolarizations to +45 mV in two cells. Cell A was exposed to 0·1 mmol l^{-1} Zn^{2+} for 10 min, and a depolarizing step was applied every minute, whereas cell B was exposed to 0·1 mmol l^{-1} Zn^{2+} for only 1 min, with pulses every 15 s. (The lack of effect after 15 s may have been partly a problem of dead space and mixing of solutions.) Zinc acts rapidly and the initial block does not appear to be use-dependent, an effect which is observed in the block of channels by local anaesthetics (Wit & Cranefield, 1974). In cell A, a steady-state response was reached after about 9 min. The full effect of 0·1 mmol l^{-1} Zn^{2+} is therefore shown by the current trace after 10 min in Fig. 2.

Effect of Zn^{2+} on Ca^{2+} currents

Ca^{2+} currents, relatively free from outward current, were recorded from cells in $0Na^+$, TEA^+ saline by depolarizing voltage pulses from a holding potential of −55 mV to potentials in the range −10 to +5 mV. One concern in these experiments is that pHi will steadily fall owing to lack of pH regulation in the Na^+-free salines. The acidosis will directly block Ca^{2+} currents (Byerly *et al.* 1984; Byerly & Moody, 1986) and contaminate the inward current owing to an increase in outward H^+ current (Byerly & Moody, 1986; Mahaut-Smith, 1989). This effect was observed, but was variable; therefore, only cells in which there was a

negligible decay in inward current following exposure to $0Na^+$, TEA^+ saline were used to study Zn^{2+} block of Ca^{2+} current. Plant & Standen (1981) may have avoided this problem in their study of Ca^{2+} currents in $0Na^+$, TEA^+ medium because 10 mmol l^{-1} 4-aminopyridine also was present. 4-aminopyridine blocks H^+ currents directly (Byerly *et al.* 1984), but can also inhibit H^+ currents indirectly (Meech & Thomas, 1987) because it is a weak base which alkalinizes the cytoplasm and increases its buffering power (Szatkowski & Thomas, 1987).

Fig. 3A shows the effect of external Zn^{2+} on the Ca^{2+} current recorded at 5 mV and in Fig. 3B the peak Ca^{2+} currents, corrected for leak current (see above for method), are plotted as a function of the membrane potential in the different levels of Zn^{2+}. Zn^{2+} blocked Ca^{2+} currents in *Helix*, but higher concentrations were required compared to the block of H^+ currents. 0·1 mmol l^{-1} Zn^{2+} caused only a small reduction in Ca^{2+} current; 1 mmol l^{-1} reduced the peak inward current and the rate and extent of its decline. This is similar to the effect of low concentrations of Cd^{2+} on calcium currents in *Helix* and *Aplysia* (Plant & Standen, 1981; Chad *et al.* 1984). Recovery of Ca^{2+} currents following exposure to

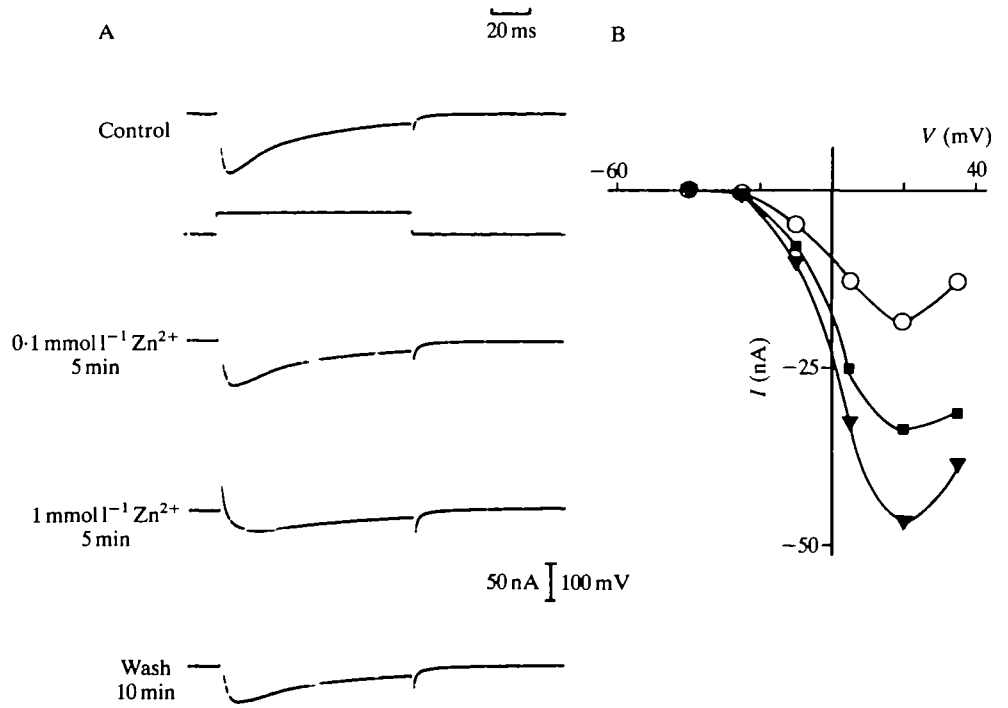

Fig. 3. Effect of Zn^{2+} on Ca^{2+} currents. (A) Voltage (lower) and current (upper) records from a cell in the control ($0Na^+$, TEA^+) saline, 5 min after exposure to 0·1 mmol l^{-1} Zn^{2+}, 5 min after exposure to 1 mmol l^{-1} Zn^{2+} and after a 10 min wash with control saline. Holding potential −55 mV. Pulse to +5 mV. Pulse length 100 ms. No change of leakage conductance throughout. (B) Current–voltage relationships. Ordinate, peak Ca^{2+} current, corrected for leak; abscissa, membrane potential during pulse. ▼, control; ■, 0·1 mmol l^{-1} Zn^{2+}; ○, 1 mmol l^{-1} Zn^{2+}.

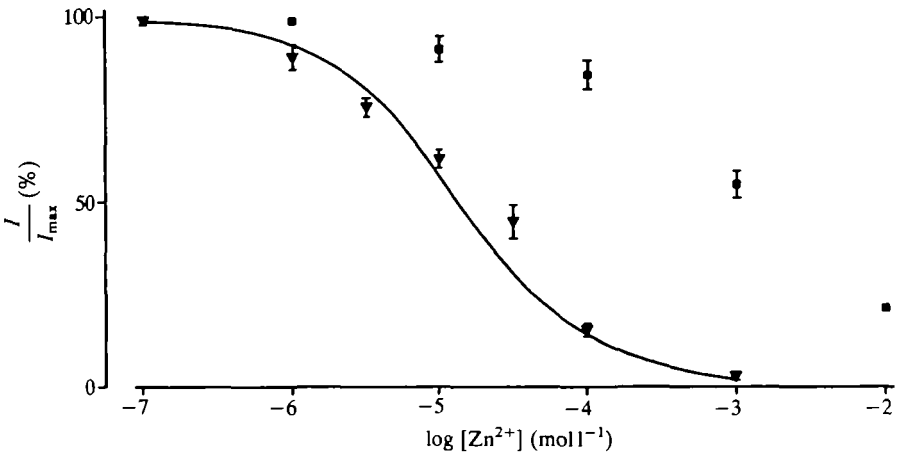

Fig. 4. Dose–response curves for block of peak Ca^{2+} and H^+ currents by external Zn^{2+}. ▼, H^+ current; ■, Ca^{2+} current; ordinate, remaining current as a percentage of the current at the start of the experiment with no blocker present. H^+ currents were measured from a 60 ms pulse to 30 mV or 45 mV (holding potential −15 mV). Ca^{2+} currents were recorded by using 45 mV or 60 mV depolarizing voltage steps from a holding potential of −55 mV. Abscissa, Zn^{2+} concentration on a logarithmic scale. Mean values are shown with standard error bars from four cells for both current types. The continuous line represents the result to be expected if one Zn^{2+} were to bind to one receptor site, where $K_d = 1{\cdot}6 \times 10^{-5}$ mol l^{-1}.

Zn^{2+} was incomplete; for example 1 mmol l^{-1} $ZnCl_2$ reduced the peak Ca^{2+} current to an average of 40 % of the control; the current then recovered to approximately 60 % after a 10 min wash.

Modelling the Zn^{2+} block

To compare fully the Zn^{2+} block of H^+ and Ca^{2+} currents, a wide range of concentrations was tested. Increasing concentrations of the blocker were added to the bath, with a short period (30–45 s) of washing between doses. Fig. 4 is a plot of the peak Ca^{2+} and H^+ current as a function of the Zn^{2+} concentration (Zn^{2+} dose on a logarithmic scale). I_{max} is the peak current measured in the absence of any blocker at the start of the experiment.

Models of blocking action are formed around the concept of the inhibitor binding to a receptor, and in turn having its specific effect. The binding reaction follows the general kinetic equation:

$$n\mathrm{B} + \mathrm{R} \underset{kn^{-1}}{\overset{kn^{1}}{\rightleftharpoons}} n\mathrm{BR}, \qquad (1)$$

where n is the number of drug molecules (B) binding to each receptor site, R. kn^1 and kn^{-1} are the reaction rates for the forward and backward reactions,

respectively. At equilibrium, the fractional occupation of receptors {[nBR]/([nBR] + [R])} is given by:

$$\frac{I}{I_{max}} = 1 - \frac{[n\mathrm{BR}]}{[n\mathrm{BR}] + [\mathrm{R}]} = \frac{1}{1 + [\mathrm{B}]/K_d}, \quad (2)$$

where I_{max} is the response in the absence of any blocker, I is the response with a blocker concentration [B] and K_d is the dissociation constant for the drug on the receptor site. When $[B] = K_d$, half the receptor sites are occupied and the response, I, is 50 %. A plot of log[B] against $\log[(I_{max}/I) - 1]$ has a slope of $1/n$ and intercepts the x axis at $\log K_d$. The experimental points for block of H^+ current were fitted best when n was close to 1, and $K_d = 1{\cdot}6 \times 10^{-5}$ mol l^{-1}. The curve in Fig. 4 shows that the data closely fit the theoretical curve for a monomolecular reaction, which suggests that each zinc ion is binding to one receptor site on the H^+ pathway. The approximate K_d for block of Ca^{2+} current was $1{\cdot}3 \times 10^{-3}$ mol l^{-1}. Ca^{2+} currents were not totally depressed by high concentrations of Zn^{2+} (up to 10 mmol l^{-1}). One reason may be that, at high concentrations, Zn^{2+} actually passes through Ca^{2+} channels (Kawa, 1979) and so no attempt was made to fit a theoretical curve to these results.

The use of Zn^{2+} to detect H^+ current contamination of Ca^{2+} current

As discussed above, recordings of Ca^{2+} currents in a few cells in $0Na^+$, TEA^+ salines slowly shifted as though an outward current were developing. This effect is shown in Fig. 5A. The lower trace was recorded 4 min after exposure to the $0Na^+$, TEA^+ saline and the upper trace 7 min later. The difference appears to be a reduction in Ca^{2+} current (the leakage conductance was not affected). pHi often falls in neurones exposed to Na^+-free salines (Mahaut-Smith, 1989); therefore, the apparent effect on the inward current could be due to direct inhibition of Ca^{2+} currents by H^+ and/or an increase in outward H^+ currents. The lower sensitivity of Ca^{2+} currents to external Zn^{2+} and the slow recovery of H^+ currents from Zn^{2+} block, allows the latter possibility to be assessed. Fig. 5B shows the currents at

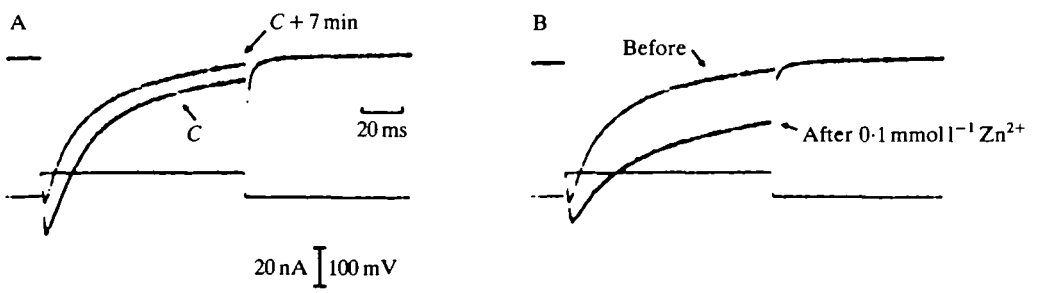

Fig. 5. Use of Zn^{2+} to block H^+ contamination of Ca^{2+} current. Membrane currents activated by voltage pulses from −55 mV to −5 mV. (A) Records are shown 4 min after exposure to $0Na^+$, TEA^+ saline (C); and 7 min later. (B) Records are shown prior to addition of Zn^{2+} and after a 3 min wash following 6 min of exposure to 0·1 mmol l^{-1} Zn^{2+}.

−5 mV before and shortly after (3 min wash) application of 0·1 mmol l^{-1} $ZnCl_2$. Clearly, although there may have been some reduction in Ca^{2+} current by acidosis, the predominant effect was caused by an increased H^+ current, which was blocked and was slow to recover from 0·1 mmol l^{-1} Zn^{2+}.

Discussion

These experiments show that low concentrations of external Zn^{2+} blocked H^+ currents in intact snail neurones. Zn^{2+} also reduced Ca^{2+} currents, but higher concentrations were required for an equivalent block. Because divalent cations have little effect on $I_{K(V)}$ current in *Helix*, Zn^{2+} provides the most selective method of inhibiting H^+ current, and is also a useful tool for investigating the physiological role of the H^+ pathway in molluscan neurones. Zn^{2+} markedly slowed H^+ current activation, which makes the block of the H^+ pathway even more effective during short depolarizations such as action potentials. Theoretically, the rapid activation of H^+ currents can cause a proton efflux within individual action potentials, which will be enhanced during bursts of activity (Byerly *et al.* 1984; Mahaut-Smith, 1989). If this pathway contributes significantly to pH regulation and/or to action potential repolarization, H^+ current block will acidify the cytoplasm and/or lengthen the duration of the action potential.

This study also demonstrates (see also Byerly & Moody, 1986; Mahaut-Smith, 1989) that H^+ currents can significantly contaminate inward Ca^{2+} current measurements. The effect is more important when pHi regulation is impaired, since acidification of the cytoplasm will increase the H^+ current. Zn^{2+} can be used to distinguish between H^+ and Ca^{2+} currents because they have very different K_d values and recovery times. The experiment of Fig. 5 showed that washing with a low concentration of Zn^{2+} blocked H^+ current and revealed underlying Ca^{2+} current.

Recently, Thomas (1988*a*) has measured pH changes at the extracellular surface of snail neurones apparently caused by efflux of H^+ through the H^+ pathway. Extracellularly applied Zn^{2+} was, not surprisingly, the most effective blocker of the surface acidification induced by membrane depolarization. 1 mmol l^{-1} Zn^{2+} completely blocked the surface acidification and the block was slow to reverse (Thomas, 1988*b*), which is very similar to the effect of this concentration of Zn^{2+} on the isolated H^+ currents in the present study.

Mechanism of action of Zn^{2+}

The mathematical fit for Zn^{2+} block of H^+ current supports the model that each zinc ion binds to one receptor site. Several agents block other ionic currents according to this monomolecular reaction, where the 'receptor' is hypothesized to be part of the channel gating mechanism or the channel itself (see Hille, 1984; for review). I have avoided the term 'channel' because, at present, there is no direct evidence that voltage-dependent H^+ movement occurs through classic ion channels. The most direct evidence for discrete H^+ channels would come from

single-channel patch-clamp experiments (Hamill *et al.* 1981). Byerly *et al.* (1984) describe the H^+ current in *Lymnaea* as a channel, presumably because H^+ current activation has many properties common to voltage-gated ionic channels. The binding model presented in this paper can only be considered as further indirect evidence that the voltage-dependent H^+ current/pathway is conducted through ion channels. The rapid effect of Zn^{2+} on the H^+ current suggests that the receptor for Zn^{2+} is situated on the extracellular membrane surface. In addition, the lack of voltage-dependence of the block supports a surface action for Zn^{2+} and not one in the voltage field.

Zn^{2+} has also been shown to block the delayed rectifier K^+ conductance of frog skeletal muscle (Stanfield, 1975). The major effect of Zn^{2+} was to slow the activation of the K^+ current by reacting with the gating particle; at $0{\cdot}1\,mmol\,l^{-1}$ Zn^{2+}, the rate constants were slowed by approximately 10-fold (Stanfield, 1975). This concentration of Zn^{2+} has a similar effect on H^+ current activation, but is more effective at reducing the steady-state H^+ conductance than the K^+ current.

The large difference in sensitivity to Zn^{2+} suggests that voltage-dependent Ca^{2+} and H^+ movements across the nerve membrane are conducted through different channels or pathways. The same pathway could carry both ions if the block is voltage-dependent and less effective in the range in which Ca^{2+} currents were activated. However, this seems unlikely as there was little difference in the block of the Ca^{2+} current activated by larger depolarizations (up to 35 mV), tested in cells with small outward currents (see, for example, Fig. 3B).

In conclusion, Zn^{2+} is the most selective inhibitor of H^+ currents known and provides a method of testing for the presence and function of the H^+ pathway.

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