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Effects of Ca^{2+} on the transient outward current of single isolated *Helix* central neurones

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1 Single *Helix* neurones were studied under voltage-clamp conditions with internal perfusion in order to examine the contribution of internal and external Ca^{2+} and the effects of 4-aminopyridine (4-AP) on the transient outward current (I_A).

2 In Na^+-free snail Ringer, replacement of external Ca^{2+} ($[Ca^{2+}]_o$) with equimolar Co^{2+} reduced the maximum amplitude of I_A and induced a shift of the steady-state part of the I_A inactivation curve (I-V curve), in a positive direction along the voltage axis when the neurone was internally perfused with K-aspartate.

3 In Ca^{2+}-free solutions, precipitation or chelation of internal Ca^{2+} ($[Ca^{2+}]_i$) by internal perfusion with KF or EGTA shifted the curve in a more negative direction without affecting the maximum amplitude of I_A. Thus, the kinetics of I_A are dependent not only upon $[Ca^{2+}]_o$, as previously suggested, but also upon $[Ca^{2+}]_i$.

4 In the presence of 4-AP the I-V curve for I_A shifted in a hyperpolarizing direction and the maximal amplitude was reduced when the neurone was internally perfused with K-aspartate. However, 4-AP had little or no effect on the I-V relationship of I_A when the neurone was internally perfused with the Ca^{2+} precipitating or chelating agent, KF or EGTA. These results suggest that the actions of 4-AP on I_A are at least partly dependent upon $[Ca^{2+}]_i$.

Introduction

The transient outward current (A current or I_A) was first demonstrated and studied in molluscan neurones (Hagiwara *et al.*, 1961; Connor & Stevens, 1971; Neher, 1971), and appears important in the regulation of cell excitability and/or subthreshold responses. Subsequently this current has also been found in mammalian central and peripheral neurones (Kostyuk *et al.*, 1981; Galvan, 1982; Gustafsson *et al.*, 1982; Belluzzi *et al.*, 1985; Oyama, 1987). It has recently been found to be regulated by Ca^{2+} and other divalent cations in molluscan and mammalian neurones (Junge, 1985; Mayer & Sugiyama, 1988), as have at least two other K^+ currents, the Ca^{2+}-activated K^+ current (I_{KCa}) (Meech & Standen, 1975) and the delayed K^+ current (I_{Kn}) (Akaike *et al.*, 1983a). However, the Ca^{2+} dependency of I_A is somewhat different from that of I_{KCa} or I_{KD}. In the experiments of Junge (1985) a neurone was perfused internally with 3 mM EGTA to determine the dependence of I_A on the external concentration of Ca^{2+} ($[Ca^{2+}]_o$). I_A was present in the EGTA-perfused neurone but subsequent replacement of $[Ca^{2+}]_o$

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with Co^{2+} reduced the amplitude of I_A. These results might be due to depletion of external Ca^{2+}. However, they might also reflect an alteration of the internal concentration of Ca^{2+} ($[Ca^{2+}]_i$).

The present experiments were designed to study the effects of changing both internal and external Ca^{2+} on I_A. In addition, we studied the effects of Ca^{2+}-chelators on the action of 4-aminopyridine (4-AP).

Methods

Experiments were performed on single identified neuronal cell bodies physically isolated from the sub-oesophageal ganglion of *Helix aspersa* (Kerkut *et al.*, 1975; Akaike *et al.*, 1978). These cells have diameters of 80 to 140 μm. The suction pipette technique (Akaike *et al.*, 1978) was used for internal perfusion and voltage-clamping, and a separate glass microelectrode was inserted to record membrane potential at times when the suction pipette was used to pass current (Brown *et al.*, 1982; Akaike *et al.*, 1983a). Ionic currents were monitored on a storage oscilloscope and recorded both on paper using a fibre optics recorder and on a digital tape recorder for subsequent analysis. The free Ca^{2+} concentration of the intracellular solution was calculated by the following equation:

$$\text{added Ca} = \frac{1 + K'([EGTA] + [Ca])}{1 + [Ca] \cdot K'} \cdot [Ca]$$

where [Ca] represents the desired free-Ca^{2+} concentration, [EGTA] represents the concentration of EGTA, and K′ is an apparent association constant for Ca^{2+}-EGTA of $10^{7.1}$ at pH 7.2 and 22°C (Portzehl *et al.*, 1964). When we used 1 mM EGTA, the Ca concentration (mM) added was 0.55, thus giving a free-Ca^{2+} concentration of 10^{-7} M. All experiments were carried out at room temperature (18–22°C).

Results

The neurones were voltage-clamped at a constant holding potential (V_H) of −40 mV throughout the experiment. Figure 1 shows the currents elicited at the same test pulse (TP) (−10 mV) before and after a hyperpolarizing prepulse (HPP) (−110 mV) in Na^+- and Ca^{2+}-free external solution (concentration in mM): Tris-Cl 85, KCl 5, $CoCl_2$ 10, $MgCl_2$ 15, glucose 5.5 and HEPES 10, pH 7.4. Without the HPP the current rose slowly and maintained its amplitude during the TP. With the HPP the current rose rapidly to a peak and then decayed via a two exponential process. Since the peak was so fast the other

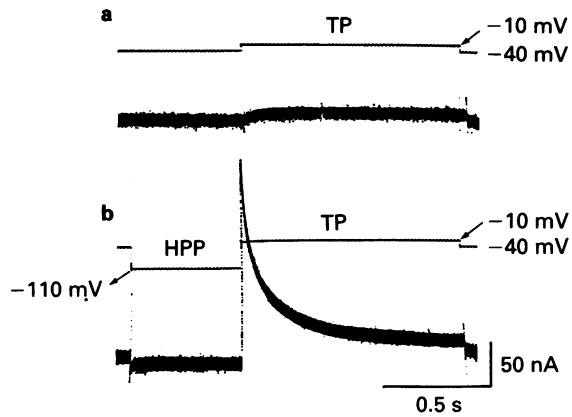

Figure 1 The currents elicited during a test pulse (TP: −10 mV) with and without a hyperpolarizing prepulse (HPP: −110 mV). (a) The current without HPP. (b) The current with HPP (the transient outward current: I_A). The holding potential was −40 mV.

currents hardly disturbed the measurement of peak I_A, and, at a TP of −10 mV, I_{KD} and I_{KCa} were negligible. The amplitude of I_A was very dependent upon the intensity and duration of the HPP. I_A was present when the HPP was 500 ms in duration and more negative than −60 mV. The amplitude increased with increasing negativity of the HPP up to −120 mV, beyond which further increases had little effect. Using a HPP of −110 mV, which produced an almost maximal response, a pulse of 100 ms duration was sufficient to activate one half of the I_A channels, while saturation of current amplitude occurred when the duration of the HPP was larger than 500 ms. On the basis of these observations we set the intensity of the TP and the duration of the HPP at −10 or −20 mV and 500 ms, respectively, for the following experiments.

Neurones were perfused with external and internal solutions for at least 30 min before experiments were started. The solutions had the following composition (in mM): the external solution, Tris-Cl 85, KCl 5, $CaCl_2$ 10, $MgCl_2$ 15, glucose 5.5 and HEPES 10, pH 7.4; the internal solution, K-aspartate 135 and HEPES 10, pH 7.2. The pH of the external and internal solutions was adjusted to 7.4 and 7.2, respectively, by adding Tris-base. The $[Ca^{2+}]_i$ was adjusted to 10^{-7} M by addition of Ca^{2+}-EGTA buffer. In the presence of $[Ca^{2+}]_o$ the steady-state inactivation curve (I-V) curve of I_A showed a threshold HPP of -52.5 ± 1.4 mV (mean ± s.e. of five experiments) (Figure 2). The midpoint of the curve was at -66.5 ± 2.3 mV (range −62 to −73 mV). Saturation of I_A amplitude occurred at HPPs more negative than −110 mV (-115 ± 5.7 mV). When

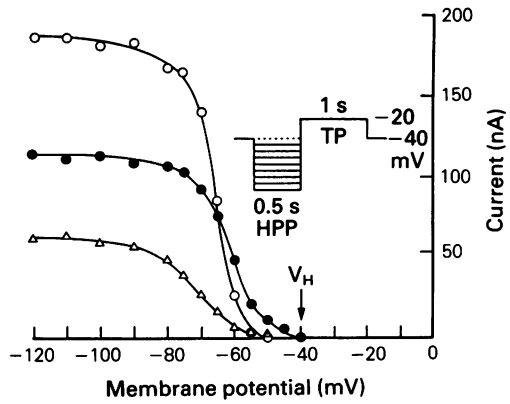

Figure 2 Effect of removal of $[Ca^{2+}]_o$ on the steady-state inactivation curve (the I-V curve) of the transient outward current (I_A). The I_A was elicited by the voltage steps, as shown in inset. The I-V curves were obtained at least 30 min after switching the external solution from control solution (○) to Ca^{2+}-free solution (●) or Ca^{2+}-free solution containing 4-aminopyridine (△). The neurone was internally perfused with K-aspartate and Ca-EGTA buffer throughout the experiment. Membrane potential on the abscissa scale indicates the potential of the hyperpolarizing prepulse (HPP). V_H = holding potential.

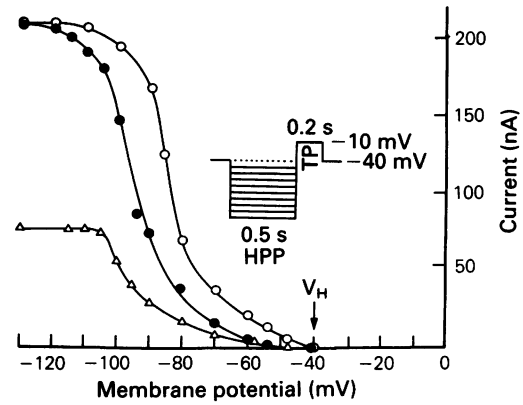

Figure 3 Effect of internal perfusion with KF on the steady-state inactivation curve (I-V curve) of I_A. The current was elicited by the voltage steps, as shown in inset. The effect on the I-V curve of I_A was examined at least 60 min after switching the internal solution from the control (○) to KF solution (●). The neurone was externally perfused with Na^+- and Ca^{2+}-free solution throughout the experiment. The action of 4-aminopyridine (4-AP) on the curve (△) was studied 30 min after the start of perfusion with the external solution containing 4-AP.

$[Ca^{2+}]_o$ was replaced with equimolar Co^{2+} (10 mM) the maximal amplitude of I_A was reduced by 42.6 ± 6.4% of control (mean of five experiments, range 27.1 to 58.3%). The midpoint of the curve also shifted by 4.3 ± 0.8 mV in a depolarizing direction, and saturation of I_A was observed at a more positive potential (−102.5 ± 5.3 mV). The threshold HPP also shifted by 3.8 ± 2.3 mV in a depolarizing direction. These shifts were probably due to a modification of the membrane surface charge by Co^{2+}. However, the reduced amplitude of I_A after removal of $[Ca^{2+}]_o$ suggests that I_A has a $[Ca^{2+}]_o$-sensitive component. These observations confirm the results of Junge (1985) on *Aplysia* neurones.

Internal application of F^- is a potent tool for precipitating Ca^{2+}; similarly internal application of EGTA leads to chelation of Ca^{2+} (Kostyuk *et al.*, 1977; Akaike *et al.*, 1982; Oyama *et al.*, 1982; Ishizuka *et al.*, 1984). Therefore, to examine the effect of $[Ca^{2+}]_i$ on I_A, the anion of the internal solution (aspartate) was totally switched to F^- (135 mM). Sixty minutes after the internal application of F^- the effect on the I-V curve was tested. As shown in Figure 3 the maximum amplitude of I_A did not change, but the potential required for saturation of I_A shifted to a more negative value, by 10.0 ± 4.1 mV (mean ± s.e. of five experiments). The midpoint of the curve ($h_{0.5}$) also shifted by 10.8 ± 2.3 mV in the same direction. The threshold HPP moved by 7.5 ± 1.4 mV to a more negative potential. A similar effect on the I-V curve was obtained by internal application of 5 mM EGTA, without altering the maximum amplitude (three experiments). These results suggest that $[Ca^{2+}]_i$ modifies the voltage-dependency of I_A.

The maximum amplitude of I_A was reduced by applying 4-AP (5 mM) externally regardless of the anion in the internal solution. The percentage inhibition of I_A by 4-AP was 54.8 ± 8.9% (mean ± s.e. of five experiments) with K-aspartate and 51.7 ± 4.7% (five experiments) with KF (Figure 3). Thus, the sensitivity of the peak I_A to 4-AP was independent of $[Ca^{2+}]_i$. However, in neurones perfused with K-aspartate, 4-AP shifted the $h_{0.5}$ by 9.8 ± 1.8 mV in a hyperpolarizing direction, while in neurones perfused with KF, 4-AP had practically no effect on $h_{0.5}$, as shown in Figures 2 and 3. Similar results were obtained in EGTA-perfused neurones (three experiments).

Discussion

The main observations from this study are as follows: (1) replacement of $[Ca^{2+}]_o$ by equimolar Co^{2+} reduced the amplitude of I_A and shifted the I-V curve in a depolarizing direction. (2) Internal

application of Ca^{2+} chelators did not produce a further decrease in the maximum amplitude of I_A in Na^+- and Ca^{2+}-free solutions, but shifted the I-V curve in a hyperpolarizing direction. (3) 4-AP shifted the I-V curve of I_A in the neurone internally perfused with K-aspartate, but not in the neurone perfused with a Ca^{2+} precipitant or chelator. It has been shown that the I_A amplitude decreases in a Ca^{2+}-free external solution in which 11 mM Ca^{2+} and 19 mM Mg^{2+} are replaced with 30 mM Co^{2+} (Junge, 1985). The possibility that the replacement of divalent cations reduces the current amplitude by modifying the membrane surface charge was considered. However, in *Helix* neurones, the efficacy of 10 mM Co^{2+} on membrane surface charge was not very great, since the I-V relationship of I_{Ca} was shifted by less than 10 mV at its peak amplitude (Akaike *et al.*, 1983a; Oyama & Kuraoka, 1986). Furthermore, in our experiments, the I-V curve of I_A shifted in a depolarizing direction by about 5 mV at the $h_{0.5}$ of the curve, while a reduction of the I_A amplitude was seen at all potentials. Therefore, the reduction in the I_A amplitude is more likely to be due to the removal of $[Ca^{2+}]_o$ than to an effect on the membrane surface charge. It appears that I_A consists of at least two components, one is $[Ca^{2+}]_o$-sensitive and the other is not.

The role of $[Ca^{2+}]_i$ in the I_{KCa} and I_{KD} is very important (Meech & Standen, 1975; Akaike *et al.*, 1983a). Therefore, it seemed worthwhile to determine if $[Ca^{2+}]_i$ still affected I_A after removal of $[Ca^{2+}]_o$. Internal application of the Ca^{2+} precipitant or chelator, F^- or EGTA, respectively, unexpectedly shifted the I-V curve of I_A in a hyperpolarizing direction along the voltage axis without affecting its maximum amplitude. One may argue that Ca^{2+} precipitants and chelators modify the surface charge of the inner membrane, resulting in a shift of the I-V curve. However, this is unlikely because in *Helix* neurones the internal application of divalent cations or Ca^{2+} chelators did not shift the I-V curves of I_{Ca} and I_{KD} (Akaike *et al.*, 1982; 1983a,b; Brown *et al.*, 1982; Akaike & Oyama, 1985). Thus, there is no evidence of any functional effects on the inner surface charge, even if they are present. Therefore, $[Ca^{2+}]_i$ seems to modify the I_A in the I-V curve directly. However, internal application of the Ca^{2+} precipitant or chelator did not produce a further decrease in the maximum amplitude of the I_A (Figure 3). Thus, the maximal amplitude of I_A after removal of $[Ca^{2+}]_o$ seems to be independent of $[Ca^{2+}]_i$.

In Ca^{2+}-free solutions, 4-AP did not shift the I-V curve of I_A when the neurone was internally perfused with KF, while it did when the neurone was perfused with K-aspartate. This suggests that $[Ca^{2+}]_i$ affects the actions of 4-AP. 4-AP has been shown to increase the I_K activated by injecting Ca^{2+} (a kind of I_{KCa}) by Hermann & Gorman (1981). Therefore, the action of 4-AP on I_A may be due to an alteration of $[Ca^{2+}]_i$.

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