BLOCKING EFFECTS OF BARIUM AND HYDROGEN IONS ON THE POTASSIUM CURRENT DURING ANOMALOUS RECTIFICATION IN THE STARFISH EGG

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SUMMARY

1. The blocking effects of Ba\(^{+}\) and H\(^{+}\) on the inward K current during anomalous rectification of the giant egg membrane of the starfish, Mediaster aequalis, were studied using voltage clamp techniques.

2. External Ba\(^{2+}\) at a low concentration (10–100 \(\mu\)M) suppresses the inward K current; the extent of suppression, expressed as the ratio of currents with and without Ba\(^{2+}\), can be described by a conventional bimolecular adsorption isotherm, \(K/(K + [Ba^{2+}]_0)\), \(K\) being an apparent dissociation constant.

3. The dissociation constant, \(K\), decreases as the membrane potential \(V\) becomes more negative and can be expressed by \(K(V) = K(0) \exp(\mu z F V/RT)\), where \(K(0)\) is the \(K\) at \(V = 0\), \(z\) is the charge of the blocking ion, and \(\mu\) is a parameter for the membrane potential dependence of Ba\(^{2+}\) blockage. The value of \(\mu\) ranges between 0.64 and 0.68.

4. Upon a sudden change in membrane potential the change in the blocking effect of Ba\(^{2+}\) follows first order kinetics; the forward rate constant is membrane-potential-dependent whereas the backward constant is potential-independent.

5. The blocking effect of Ba\(^{2+}\) appears to be independent of the activation of K channels during anomalous rectification.

6. The blocking effect of Ba\(^{2+}\) depends on \(V\) alone, in contrast to the activation of the K channel during anomalous rectification which depends on \(V - V_K\).

7. In these respects, the effect of Ba\(^{2+}\) is equivalent to the introduction of inactivation into the anomalous rectification.

8. Sr\(^{2+}\) and Ca\(^{2+}\) show small but observable blocking effects only at much higher concentrations (about 10–20 mM).

9. The inward K current is suppressed when the external pH is reduced below 6.0. The blocking effect of H\(^{+}\) shows no significant potential dependence. The concentration dependence suggests that three H\(^{+}\) ions simultaneously titrate the acidic groups of each channel (pK = 5.3–5.4).

10. The implications of these results are discussed in terms of molecular models of the potassium channel of anomalous rectification and possible mechanisms of K channel inactivation.
INTRODUCTION

The egg cell of the starfish shows anomalous, or inward, rectification (Hagiwara & Takahashi, 1974; Miyazaki, Ohmori & Sasaki, 1975; Hagiwara, Miyazaki & Rosenthal, 1976). Its properties are similar to those of the anomalous rectification found in frog skeletal muscle (Katz, 1949; Hodgkin & Horowicz, 1959; Adrian & Freygang, 1962a,b; Adrian, 1964, 1969; Adrian, Chandler & Hodgkin, 1970; Almers, 1971). Under normal conditions this membrane current is carried by K+ ions through a channel which is sensitive to membrane potential and external K+ concentration. Some monovalent cations such as Cs+, Rb+ and Tl+ reduce the K+ currents by blocking the channel when they are introduced into the external solution (Hagiwara & Takahashi, 1974; Hagiwara et al. 1976; Hagiwara, Miyazaki, Krasne & Ciani, 1977). Na+, Cs+ and Rb+ have also been shown to block the K channel responsible for the outward rectification when introduced internally in the squid axon (Bezanilla & Armstrong, 1972; French & Adelman, 1975; French & Wells, 1977). Ba2+ is known to be a potent blocker of the K channel of the outward rectification when applied internally to squid axon (D. C. Eaton & M. S. Brodwick, 1977, personal communication), and in vertebrate (Sperelakis, Schneider & Harris, 1967) and invertebrate muscle fibres (Werman & Grundfest, 1961; Werman, McCann & Grundfest, 1961). H+ ions block various ionic channels in the nerve membrane, probably by titrating acidic sites within the channel (Hille, 1968).

The present work investigates the blocking effects of both Ba2+ and H+ ions on the K channel of the anomalous rectification by using voltage clamp techniques. The giant egg (1.0–1.2 mm in diameter) of the starfish, Mediaster aequalis, was used for this study. The experimental results show that both Ba2+ and H+ in the external medium block the channel at very low concentrations. The blocking effect of Ba2+ is membrane potential dependent; the steady-state blocking effect indicates that one Ba2+ ion affects each channel. In contrast, no significant membrane potential dependence is found for the effect of H+; the concentration dependence of the block suggests that three H+ ions simultaneously titrate the acidic groups of each channel.

METHODS

Immature eggs of the starfish, Mediaster aequalis, were used. The collection of eggs and experimental techniques were similar to those described previously (Hagiwara et al. 1976).

The composition of the major solutions is shown in Table 1. Most of the experiments were performed in solution B (25 mM-K+). An appropriate amount of 10 mM-BaCl2 solution was added to the solution to obtain final Ba2+ concentrations of 3–300 μM. The Ca2+ concentration was varied by mixing solutions D and E in appropriate proportions. For experiments with Sr2+ or Mn2+, the CaCl2 of solution E was replaced by SrCl2 or MnCl2. The effect of varying the pH was examined at 25 mM-K+ and the composition (mM) of the solution was: NaCl, 385; KCl, 15; CaCl2, 10; MgCl2, 50; K biphthalate, 10; acetic acid, 10; HEPES, 10; Tris-maleate, 10; glycine, 10. The pH of the solution was adjusted to 4–8 with NaOH. Experiments were performed at room temperature (21–22 °C).

RESULTS

The resting membrane of the starfish egg behaves as an almost perfect K+ electrode when the external K+ concentration is altered (Hagiwara & Takahashi, 1974). Since K+ is the major site inside the cell (Shen & Steinhardt, 1976), the resting,
Table 1. Composition of solutions

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>KCl</th>
<th>NaCl</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>474</td>
<td>10</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>459</td>
<td>10</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>434</td>
<td>10</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>474</td>
<td>0</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>25</td>
<td>399</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

The pH of the solution was adjusted either to 7.7 with 5 mm-Tris-OH HCl or to 7.0 with 5 mm-HEPES-NaOH.

or zero-current, membrane potential (V₀), probably represents the K⁺ equilibrium potential (V_K). The K conductance of the membrane shows an anomalous or inward rectification. According to the previous work (Hagiwara et al. 1976) the rectification has instantaneous and time-dependent components. The time-dependent increase in the K conductance for the negative voltage pulse as well as the decrease in the conductance for the positive pulse follows first-order kinetics. Records marked as 0 µM in Fig. 1 illustrate the time course of the current for the membrane clamped at various potentials from the zero-current potential in 25 mM-K⁺ in the absence of Ba²⁺ (solution B). The initial capacity transient is not seen in the records. Each trace starts with the instantaneous current I₀ followed by the time-dependent increase of the current to a steady-state value, Iₛ. The total membrane current I during the voltage pulse is expressed by

\[ I = Iₛ - (Iₛ - I₀) \exp \left( -t/\tau \right) \]  \hspace{1cm} (1)

\( \tau \) being the time constant of the time-dependent component. Previous work (Hagiwara et al. 1976) has shown that the activation of the inward K channel does not depend on V alone, but rather on \( V - V_K \). The steady-state K conductance expressed by \( Iₛ/(V - V_K) \) decreases as the membrane potential is made more positive and becomes almost negligible at \( V - V_K = 30 \) mV, and increases as the potential becomes more negative, reaching saturation at about \( V - V_K = -40 \) mV.

When the negative voltage pulse is relatively small, the membrane current during the entire 4-5 sec of the voltage pulse (traces -10, -18 and -25 mV) is expressed accurately by eqn. (1) which describes the activation of the channel without inactivation. When the membrane potential during the pulse becomes more negative, however, the current declines slowly after reaching a maximum (trace -32 mV); the rate of decline tends to increase as the membrane potential becomes
more negative. It is thus necessary to introduce an inactivation term to describe the entire time course of the current more accurately. However, the amount of decline at the end of a 4–5 sec voltage pulse is small, and almost negligible when compared with that of the decline observed with Ba\(^{2+}\) (see below). Since inactivation of the inward current is so pronounced in Ba\(^{2+}\)-containing solutions, its time course and voltage dependence can be readily studied. Combining this data with dose–response curves for the effect of Ba\(^{2+}\) on the inward current, some proposals can be made as to the mechanism of the slight inactivation of the inward K current seen in normal bathing solutions.

Records in Fig. 1 were obtained from a cell in 25 mM-K\(^+\) with 0, 30, 100, and 300 \(\mu}\text{M-BaCl}_2\), respectively. These small concentrations of Ba\(^{2+}\) resulted in a drastic change in the behaviour of the membrane current: (1) the maximum amplitude that the current reached during the pulse to a given potential decreased with increasing Ba\(^{2+}\) concentration; and (2) the membrane current declined during the voltage pulse, attaining a steady-state value which also decreased with increasing Ba\(^{2+}\) concentration. The instantaneous current–voltage relation (not shown in the Figure) obtained by two-step voltage-clamp experiments indicates that the decline of the current is due to a decrease in the membrane conductance, not to a change in the driving force. Ba\(^{2+}\) appears to reduce the inward K current by blocking the K channel of the inward rectification. As will be shown below, the extent of the Ba\(^{2+}\) block is a function of both membrane potential and time: the relaxation of the current from the peak to the steady-state value during a constant voltage pulse represents the time-dependent onset of the Ba\(^{2+}\) block appropriate to the new membrane potential.

In order to analyse the extent of the block by Ba\(^{2+}\), parameter \(h\) is introduced and the current \(I'(V, t)\) in the presence of Ba\(^{2+}\) is expressed by

\[
I'(V, t) = hI(V, t).
\]  

(2)

\(I(V, t)\) is the current in the absence of Ba\(^{2+}\) obtained at the same voltage and time. Therefore \(h\) is the normalized current. The parameter \(h\) is a function of voltage and time but is tentatively assumed independent of the activation of the channel. Under this condition the parameter \(h\) resembles the inactivation factor, \(h_\infty\), of the Na channel described in the original Na theory (Hodgkin & Huxley, 1952). The effect of Ba\(^{2+}\) is then considered analogous to the introduction of inactivation into the inward rectification.

When the membrane is held at the zero-current potential \((V_0)\), \(h\) has its steady-state value at \(V_0\), given by \(h_\infty(V_0)\). If the membrane potential is shifted to a more negative value \(V\), \(h\) will decrease to a new steady-state value, \(h_\infty(V)\), in a time dependent fashion. The change from \(h_\infty(V_0)\) to \(h_\infty(V)\) is recorded as a time dependent decrease in the current. Measurement of the current voltage relation at different times after the shift in membrane potentials should yield information as to the state of the \(h\) variable. Measurements at the beginning of a voltage pulse give information about \(h_\infty(V_0)\), whereas the steady-state values pertain to \(h_\infty(V)\).

Steady-state current–voltage relations

The membrane current declined and reached a steady-state amplitude in 4–5 sec when \([\text{Ba}^{2+}]_0\) was greater than 30 \(\mu\text{M}\) (Fig. 1). Steady-state current–voltage relations were obtained at the end of a 4–5 sec voltage pulse from \(V_0\) (–51 mV) in 25 mM-K\(^+\)
when the external solution contained 0, 30, 100 and 300 μM-BaCl₂ (Fig. 2A). The current at 4-5 sec was normalized to that obtained in the absence of Ba²⁺ at the same potential and time. This was considered \( h_\infty(V) \) and plotted in Fig. 2B against the membrane potential during the voltage pulse. For a given membrane potential \( V \), \( h_\infty(V) \) decreased with increasing Ba²⁺ concentration and for a given Ba²⁺ concentration \( h_\infty(V) \) decreased as \( V \) becomes more negative. The above experimental result can be explained by assuming that the blockage of the K channel follows a

\[
\begin{align*}
\text{Fig. 2.} & \ A, \text{current–voltage relations at the steady state reached at the end of a 4-5 sec voltage pulse at different Ba²⁺ concentrations (listed next to each curve). B, } I_{\text{with Ba²⁺}} / I_{\text{without Ba²⁺}} \text{ plotted against } V (\text{filled circles). Open circles and crosses represent normalized currents at 150 msec from the onset of the voltage pulses of -70 and -80 mV respectively plotted at } V = -51 \text{ mV } (V_0). \text{ Continuous curves were drawn from eqns. (3) and (4) with } K(0) = 560 \mu M \text{ and } \mu = 0.64. A \text{ and } B \text{ were obtained from the same cell at } 25 \text{ mM-K⁺; holding potential } V_0 = -51 \text{ mV.}
\end{align*}
\]

conventional bimolecular adsorption isotherm with a membrane potential dependent dissociation constant, i.e.

\[
h_\infty(V) = \frac{K(V)}{K(V) + [\text{Ba}^{2+}]_0}, \quad (3)
\]

where

\[
K(V) = K(0) \exp \left( \frac{z\mu F V}{RT} \right). \quad (4)
\]

Here, \( K(0) \) is the dissociation constant at 0 membrane potential, \( z \) is the charge of the blocking ion (\( z = 2 \) in the present case), \( RT/F \) is 25 mV at 22 °C and \( \mu \) is a parameter for the membrane potential dependence of Ba²⁺ blockage. Continuous lines in Fig. 2B were drawn using eqns. (3) and (4), assuming that \( K(0) = 560 \mu M \)
and $\mu = 0.64$. The experimental data show good agreement with the calculated curves. The parameter $\mu$ deduced for three other preparations was 0.68, 0.65 and 0.64.

**Current–voltage relations at 150 msec**

According to the assumptions presented, the extent of the $\text{Ba}^{2+}$ blockage at the holding potential, $V_0$, should also be described by eqns. (3) and (4). If $h$ changes with a time course much slower than that of the activation of the inward rectification, values of $h_\infty(V_0)$ can be assayed by observing the current–voltage relation at the beginning of the voltage clamp pulse. Current–voltage relations at 150 msec after the onset of the pulse from $V_0$ ($-51$ mV) were obtained with the cell used in Fig. 2. Normalized currents measured at $V_{\text{test}} = -70$ mV (open circles) and $-80$ mV (crosses) were plotted in Fig. 2B for $V = -51$ mV which was the holding potential of this experiment. These points are reasonably fitted by the calculated curves. This result suggests that at 150 msec from the onset of the voltage pulse the value of $h$ has not yet been changed significantly from its steady-state value at the holding potential $V_0$.

![Fig. 3. A, current–voltage relations at 150 msec from the onset of the voltage pulse measured in different $\text{Ba}^{2+}$ concentrations (listed for each curve). B, $I_{\text{with } \text{Ba}^{2+}}/I_{\text{without } \text{Ba}^{2+}}$ obtained at $V = -70$, $-80$ or $-90$ mV plotted against $[\text{Ba}^{2+}]_0$. The continuous curve was drawn from eqn. (3) with the dissociation constant $K(V_0) = 27 \mu\text{M}$. A and B were obtained with the same cell at 25 mM-K$^+$; holding potential $V_0 = -50$ mV.](image-url)
In the experiment of Fig. 3 A current-voltage relations at 150 msec from the onset of the voltage pulse from \( V_0 \) (-50 mV) were obtained in 25 mM-K\(^+\) in a different cell when the Ba\(^{2+}\) concentration was varied from 3 to 300 \( \mu \)M. The normalized current is plotted against \([\text{Ba}^{2+}]_o\) in Fig. 3 B. Data obtained at \( V = -70, -80, \) and \(-90\) mV are almost identical and this confirms again that 150 msec is too short a time for the potential dependence of the blocking effect to develop. Thus, Fig. 3 B is considered to represent \( h_\infty(V_0) \) as a function of Ba\(^{2+}\). The continuous curve has been drawn according to eqn. (3) with \( K(V_0) = 27 \mu \)M. Similar experiments were performed in two other cases in 25 mM-K\(^+\) and \( K(V_0) \) was 24 and 30 \( \mu \)M.

**Effect of the holding potential**

In the preceding experiments the holding membrane potential \( (V_{\text{hold}}) \) was always kept at the zero-current potential \( V_0 \). The curves in Fig. 2 B predict that \( h_\infty \) would increase when \( V_{\text{hold}} \) is made more positive than \( V_0 \). In the experiment illustrated by Fig. 4 A the membrane potential was first held at various levels more positive than \( V_0 \) (-51 mV) and then shifted to a fixed large negative level (-90 mV for 30 and 100 \( \mu \)M-Ba\(^{2+}\), and \(-93\) mV for 300 \( \mu \)M-Ba\(^{2+}\)) to activate the inward rectification. Current traces for different holding potentials are superimposed in each set of records. Since outward rectification is almost absent for the membrane potential more negative than +20 mV (see Fig. 2 A, and Hagiwara, Ozawa & Sand, 1975) only small outward currents are required to hold the membrane at those positive levels. These outward holding currents are seen at the beginning of each set of records in Fig. 4 A. In response to the test pulse the inward current develops to the peak and then declines to the steady-state level. The amplitude of the inward current is a function of \( V_{\text{hold}} \). Although it is not shown in the Figure the inward current during the test pulse obtained in the absence of Ba\(^{2+}\) is independent of \( V_{\text{hold}} \).

The amplitude of the peak inward current during the test pulse increases as \( V_{\text{hold}} \) becomes more positive and finally reaches a saturation value which depends upon \([\text{Ba}^{2+}]_o\). Although the amplitude of the peak current depends on \( V_{\text{hold}} \), the time course of the current during the test pulse is independent of \( V_{\text{hold}} \), and depends only on the membrane potential during the test pulse, \( V \), when \([\text{Ba}^{2+}]_o\) is fixed. When the peak amplitude reaches a saturation value at a positive \( V_{\text{hold}} \), we assume that no blocking effect of Ba\(^{2+}\) is present, i.e. \( h_\infty = 1 \). The amplitude of the peak current at this \( V_{\text{hold}} \) is still smaller than the maximum inward current obtained with a test pulse reaching the same membrane potential in the absence of Ba\(^{2+}\). Although the potential dependent change of \( h \) from \( h_\infty(V_{\text{hold}}) \) to \( h_\infty(V) \) during the test pulse \( V \) proceeds slowly, it probably becomes significant before complete activation of the inward rectification occurs, i.e. \( h \) at the time of peak current is smaller than \( h_\infty(V_{\text{hold}}) \). As mentioned above the time course of the inward current depends only on the membrane potential, \( V \), during the test pulse and is independent of \( V_{\text{hold}} \). The amplitude as well as the time course of the inward current obtained in the absence of Ba\(^{2+}\) is independent of \( V_{\text{hold}} \). Eqn. (2) indicates that the time course of \( h \) from \( h_\infty(V_{\text{hold}}) \) to \( h_\infty(V) \) is also independent of \( V_{\text{hold}} \). When the time course is expressed by \( f_v(t) \) the change of \( h \) can be described by

\[
h = h_\infty(V_{\text{hold}}) - \{h_\infty(V_{\text{hold}}) - h_\infty(V)\} f_v(t),
\]

(5)

the value of \( f_v(t) \) changes from 0 to 1 with time. When \( f_{v,\text{peak}} \) represents the value of
$f_v(t)$ at the time of the peak inward current, the value of $h^*$ at the peak of the inward current is given by

$$h^* \approx (1 - f_{v,\text{peak}}) h_{\infty}(V_{\text{hold}}),$$

since $h_{\infty}(V_{\text{hold}}) \gg h_{\infty}(V)$ and $f_{v,\text{peak}} \ll 1$. Therefore, $h_{\infty}(V_{\text{hold}})$ is given by the ratio of the peak amplitude of the inward current, $I_p$, at $V_{\text{hold}}$ and $I_{p,\text{max}}$ at the saturation level.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{A, effect of the holding potential upon the inward K current obtained at different Ba$^{2+}$ concentrations. The potential during the test voltage pulse was $-90$ mV for 30 $\mu$M and 100 $\mu$M-Ba$^{2+}$ and $-93$ mV for 300 $\mu$M-Ba$^{2+}$. Interrupted lines represent the zero current level. The ‘holding’ membrane potential, $V_{\text{hold}}$, is listed for each current trace. B, potential dependence of the steady-state blocking effect of Ba$^{2+}$. The peak amplitude, $I_{\text{peak}}$, of the current during a test pulse to $-80$ mV (filled circles) or $-90$ mV (open circles) is normalized with the maximum peak amplitude, $I_{p,\text{max}}$, measured at a large positive holding potential and the normalized value is plotted against the holding potential. Continuous lines were calculated from eqns. (3) and (4) with $K(0) = 560$ $\mu$M and $\mu = 0.64$. A and B were obtained from the same cell, $[K^+]_o = 25$ mM and $V_o = -51$ mV.}
\end{figure}

at which $h_{\infty} = 1$. The value of $I_p/I_{p,\text{max}}$ obtained with test pulses to membrane potentials $-80$ and $-90$ mV are plotted against $V_{\text{hold}}$ in Fig. 4B. Continuous lines were drawn according to eqns. (3) and (4) for $K(0) = 560$ $\mu$M and $\mu = 0.64$. Data in Figs. 2 and 4 were obtained from the same cell in 25 mM-K$^+$. The curves in these two Figures are identical. The results indicate that the extent of the Ba$^{2+}$ blockage
can be described by simple bimolecular kinetics over a wide range of potentials when measured as a function of either the steady-state potential or the holding potential.

The assumption that \( h_\alpha(V_{\text{hold}}) = 1 \) at the saturation level of \( I_p \) is justified by the following observation. As described in the next section the falling phase of the inward current during the test pulse is well fitted by a single exponential curve. During this period the current response to a pulse to the same membrane potential has already reached a final steady amplitude, \( I_v \), in the absence of \( \text{Ba}^{2+} \). Therefore \( I_\alpha h_\alpha(V_{\text{hold}}) \) can be estimated as the current amplitude at \( t = 0 \) by the extrapolation of the exponential decay of the current. Since \( I_v \) is known, \( h_\alpha(V_{\text{hold}}) \) can be estimated. The value of \( h_\alpha(V_{\text{hold}}) \) at saturation of \( I_p \) was calculated and was very close to unity for all three \( \text{Ba}^{2+} \) concentrations.

\[ V = -81 \text{ mV} \]

\[ [\text{Ba}^{2+}]_0 = 100 \mu\text{M} \]

\[ 10^{-7} \]

\[ 10^{-8} \]

\[ 100 \mu\text{M} \]

\[ 300 \mu\text{M} \]

\[ T \]

\[ V_{\text{cond}} = -30 \text{ mV} \]

\[ V_{\text{cond}} = -51 \text{ mV} \]

\[ -10 \text{ mV} \]

\[ 0.1 \mu\text{A} \]

\[ V_{\text{test}} = -70 \text{ mV} \]

Fig. 5. \( A \) and \( B \), time course of membrane current \( (A) \) during a voltage pulse to \( V, [I_\alpha(V) - I(V, t)] \) is plotted against \( t \) on a logarithmic scale. \( V \) is fixed at \(-81 \text{ mV} \) and \( [\text{Ba}^{2+}]_0 \) is varied in \( A \), \( [\text{Ba}^{2+}]_0 \) is fixed at \( 100 \mu\text{M} \) and \( V \) is varied in \( B \). \( [K^+]_0 = 25 \text{ mM} \), holding potential \( V_0 = -50 \text{ mV} \). \( C \), the time course of blocking effect at membrane potentials more positive than \( V_0 \). The holding potential was \(-10 \text{ mV} \). The membrane potential was then shifted to a conditioning level \( V_{\text{cond}} \) (\(-30 \text{ mV} \) for the upper trace and \(-51 \text{ mV} \) for the lower trace). After different durations of the conditioning pulse a test pulse to \(-70 \text{ mV} \) was applied. Currents obtained with different durations of the conditioning pulse are superimposed. Interrupted lines represent the zero-current level. \( [K^+]_0 = 25 \text{ mM}, [\text{Ba}^{2+}]_0 = 300 \mu\text{M} \) and \( V_0 = -51 \text{ mV} \).

**Time course of Ba blocking**

When the external solution contained barium ions the membrane current, \( I(V, t) \), decayed during the voltage pulse and reached a final value, \( I_\alpha(V) \), in 4–5 sec (Fig. 1).
Since the decline in current in the absence of Ba is almost negligible in the ranges of membrane potential and time examined in this experiment, the normalized current should show an identical rate of decay. In other words, the time course of the decline of membrane current can be considered that of the change in \( h \). \( [I_\infty(V) - I(V, t)] \) is plotted against \( t \) on a logarithmic scale in Fig. 5\( A \) and \( B \); the plot shows that the decline of the membrane current is fitted very well to a single exponential curve. At a fixed membrane potential, the time constant \( \tau \) decreases as \([\text{Ba}^{2+}]_0\) increases (Fig. 5\( A \)); at a fixed \([\text{Ba}^{2+}]_0\), \( \tau \) decreases as \( V \) becomes more negative (Fig. 5\( B \)). Time constants at different membrane potentials were calculated at 30, 100, and 300 \( \mu \text{M-Ba}^{2+} \) and plotted against \( V \) on a logarithmic scale in Fig. 6\( A \).

![Graph]

Fig. 6. \( A \), time constant, \( \tau \), of the decay of the inward current during the voltage pulse obtained at three different \( \text{Ba}^{2+} \) concentrations plotted against the membrane potential on a logarithmic scale. \([\text{K}^{+}]_0 = 25 \text{ mm}, \) holding potential \( V_0 = -51 \) to \(-53 \text{ mV} \). The three different symbols refer to three different cells. Open circles represent the time constant of the decrease in the peak inward current measured as in Fig. 5\( C \). Data illustrated by open and filled circles were obtained from the same cell. \( B \) and \( C \), \( (1-h_{\infty})/\tau \) and \( h_{\infty}/\tau \) are plotted against the membrane potential. Continuous curves were drawn according to eqns. (12) and (13) with \( b = 189 \text{ sec}^{-1} \text{ m}^{-1} \) and \( a = 0.11 \text{ sec}^{-1} \). \([\text{K}^{+}]_0 = 25 \text{ mm} \).

The above data indicate that the blockage of the K channel by barium follows first order kinetics; the blocking reaction can be written

\[
\text{channel + Ba}^{2+} \xrightleftharpoons[\alpha]{\beta} \text{blocked channel},
\]

where the reaction rates are in principle voltage-dependent. The change in \( h \) following a voltage step from \( V_0 \) to \( V \) can be consequently deduced by integration of the equation

\[
\frac{dh}{dt} = \alpha(V)(1-h) - \beta(V) \cdot [\text{Ba}^{2+}]_0 \cdot h,
\]  
(7)
BLOCKING BY $\text{Ba}^{2+}$ AND $H^+$

which gives

$$h(t) = h_\infty + (h_0 - h_\infty) e^{t/\tau},$$

where

$$\frac{1}{\tau} = \alpha(V) + \beta(V) \cdot [\text{Ba}^{2+}]_0,$$

$$\alpha(V) = \frac{h_\infty}{\tau},$$

$$\beta(V) \cdot [\text{Ba}^{2+}]_0 = \frac{1 - h_\infty}{\tau}. \quad (11)$$

Since $h_\infty$ and $\tau$ have been obtained experimentally actual values of $h_\infty/\tau$ and $(1 - h_\infty)/\tau$ can be calculated. Calculated data are plotted against $V$ for three different $\text{Ba}^{2+}$ concentrations (Fig. 6B and C). The result indicates that $\alpha$ is independent of $V$ whereas $\beta$ depends on $V$. The calculated values are expressed by

$$\frac{1 - h_\infty}{\tau} = b[\text{Ba}^{2+}]_0 \exp \frac{-z\mu F V}{RT}, \quad (12)$$

$$\frac{h_\infty}{\tau} = a, \quad (13)$$

where $\mu$ is a parameter determined from the steady-state blocking which is equal to 0.64 for this cell and $a$ and $b$ are constants specific to each cell.

Although many models could account for such results, the simplest is probably the one based on the following two assumptions. (1) The binding site for $\text{Ba}^{2+}$ is located somewhere within the channel, where the average potential drop from the external solution is about 64% ($\mu$) of the total transmembrane potential, i.e. $V_{\text{site}} \approx 0.64 \, V$. (2) The peak of the activation energy barrier for translocation of $\text{Ba}^{2+}$ from the external solution to the binding site is immediately adjacent to the site. In this case, the potential drop between the peak of the barrier and the site is negligibly small and, according to Eyring rate theory, the rate constant for the backward movement becomes practically independent of the membrane potential.

Fig. 4 indicates that significant steady-state blocking by barium exists even at positive membrane potentials ($V - V_0$ greater than $+30 \, mV$) at which, in the absence of $\text{Ba}^{2+}$, practically all of the K channels are closed. The time course of the Ba blocking at such membrane potentials was studied by using a two-step voltage-clamp technique (Fig. 5C). In this experiment the membrane potential was first held at $-10 \, mV$ and then shifted to a conditioning level. The value of $h$ should change from $h_\infty (-10 \, mV)$ to $h_\infty(V_{\text{cond}})$ during the conditioning pulse. However, this is not seen in the current recording since most of the inward K channels are non-conductive at this conditioning potential. In order to examine the time course of $h$ the second pulse was applied to bring the membrane potential to $-70 \, mV$ at which the inward K channel is activated. Traces obtained at various durations of the conditioning pulse are superimposed in Fig. 5C. The peak amplitude of the inward current during the test pulse decreases as the duration of the conditioning pulse increases. The time course of $h$ was determined by plotting the peak current against the duration of the conditioning pulse. Time constants were then estimated and plotted against the conditioning potential with open circles in Fig. 6A.

The result shown by Fig. 6A indicates that $\tau$ increases linearly with $h_\infty$ obtained
from the steady-state blocking effect. Continuous lines in Fig. 6A were drawn by assuming that the ratio between \( \tau \) and \( k_{\infty} \) is constant. The best fit was made simply by eye. Dashed lines were obtained as extrapolations of the continuous curves. The time constants observed at more positive membrane potentials (open circles in Fig. 6A) agree well with values estimated by extrapolation. It therefore appears that the conductive or nonconductive state of the K channel does not affect the kinetics of the Ba\(^{2+}\) block.

![Graph](image)

**Fig. 7.** A, membrane currents during voltage clamp at 25 and 50 mM-K. The upper set of traces was obtained without Ba\(^{2+}\) and the lower set with 100 \( \mu \)M-Ba\(^{2+}\). The holding potential was \( V_0 = -51 \text{ mV} \) for 25 mM and \(-31 \text{ mV} \) for 50 mM-K\(^+\). B, the membrane potential dependence of the steady-state blocking effect of 100 \( \mu \)M-Ba\(^{2+}\) was obtained at 10, 25 and 50 mM-K\(^+\) by observing the peak current during the test pulse \((V - V_0 = -40 \text{ mV})\) while the holding potential was altered as in the case of Fig. 4A. \( V_0 = -70 \text{ mV} \) at 10 mM-K\(^+\), \(-51 \text{ mV} \) at 25 mM-K\(^+\) and \(-31 \text{ mV} \) at 50 mM-K\(^+\). The normalized currents obtained at 125 msec from the onset of the voltage pulse \((V - V_0 = -30 \text{ mV})\) when the holding potential was \( V_0 \) are also plotted at \(-70 \text{ mV} \) (for 10 mM-K\(^+\)), \(-51 \text{ mV} \) (for 25 mM-K\(^+\)) and \(-31 \text{ mV} \) (for 50 mM-K\(^+\)) with filled stars.
**Effect of K⁺ concentration on Ba²⁺ blocking**

Fig. 7A shows membrane currents obtained at 25 and 50 mM-K⁺ with and without 100 μM-Ba²⁺. The holding membrane potential was the zero current level (−51 mV at 25 mM-K⁺, and −31 mV at 50 mM-K⁺). The records suggest that the blocking effect of Ba²⁺ for a given V − V₀ is substantially less at 50 mM-K⁺ than at 25 mM-K⁺. Records in 10 mM-K⁺ (V₀ = −70 mV) are not shown in the Figure but the effect at 10 mM-K⁺ was greater than at 25 mM-K⁺. In order to quantify the results, current-voltage relations at 125 msec were determined at 10, 25 and 50 mM-K⁺ with and without 100 μM-Ba²⁺ and the normalized current at V − V₀ = −30 mV was calculated. It was 0-08 at 10 mM-K⁺, 0-24 at 25 mM-K⁺ and 0-41 at 50 mM-K⁺. V − V₀ = −20 or −40 mV gave a set of very similar values. The results appear to indicate that the blocking effect of Ba²⁺ decreases substantially as the K⁺ concentration increases. This apparent decrease is, however, primarily due to the fact that the increase in the K⁺ concentration results in a positive shift of the holding potential which is the zero-current potential V₀. For each K⁺ concentration the effect of V_hold was examined by measuring I_p/I_p.max just as in Fig. 4B. The results (Fig. 7B) show that the data at different K⁺ concentrations can be fitted by a single curve. The continuous line was drawn from eqns. (3) and (4) assuming that K(0) = 590 μM and μ = 0-68. The normalized currents obtained at 125 msec when V_hold = V₀ at the three different K⁺ concentrations are also plotted in the same Figure (filled stars). They coincide with the same calculated curve. Therefore, it is safe to conclude that the apparently smaller blocking effect of Ba²⁺ at higher K⁺ concentrations is due primarily to the positive shift of V₀ at higher K⁺ concentrations. This, in turn, indicates that the dissociation constant, K(0), in eqn. (4) is independent of the external K⁺ concentration.

**Other divalent cations**

The foregoing results demonstrate that Ba²⁺ blocks the K⁺ current of the inward rectification at very small concentrations. Neither Sr²⁺ nor Ca²⁺ at comparable concentration resulted in any observable effect; at concentrations above 5 mM, however, Sr²⁺ and Ca²⁺ showed a small but significant effect. Fig. 8A shows current-voltage relations at 150 msec from the onset of the voltage pulse in 25 mM-K⁺. The external solutions contained 5, 10 or 20 mM of either CaCl₂ or SrCl₂. The Mg²⁺ concentration was kept at 50 mM throughout. I_{150msec} at a given V decreases with increasing concentration and this is more marked in Sr²⁺ than in Ca²⁺. In order to determine whether eqn. (3) holds for Sr²⁺ and Ca²⁺, the reciprocal of I_{150msec} was plotted against the concentration for V equal to −70 and −80 mV in Fig. 8B; in each case, the points can be fitted by a straight line. The straight lines for Ca²⁺ and Sr²⁺ intercept the Y-axis at approximately the same point at each potential. This indicates that the blocking effect of Ca²⁺ and Sr²⁺ can be described by eqn. (3). The dissociation constants obtained at the two different potentials are similar: K_{Sr} = 9-10 mM and K_{Ca} = 25–30 mM. The dissociation constant for Ba²⁺ under comparable conditions (25 mM-K⁺, V_hold = V₀ = −50 to −53 mV) was between 24 and 30 μM. However, this value was obtained in the presence of 10 mM-Ca²⁺. K_Ba in the absence of Ca²⁺ can be calculated if one assumes that Ca²⁺ and Ba²⁺ compete for the same site. Taking K_{Ca} = 25 mM, K_Ba in the absence of Ca²⁺ ranges between
17 and 21 μM. This indicates that the affinity for Ba\(^{2+}\) is greater than that for Ca\(^{2+}\) and Sr\(^{2+}\) by a factor of 10\(^3\). A few experiments were performed with Mn\(^{2+}\) and the results suggest that the blocking effect of Mn\(^{2+}\) is less than that of Ca\(^{2+}\). The effect of Mg\(^{2+}\) was not studied. However, since the effect of 5–20 mM-Ca\(^{2+}\) was observable in the presence of 50 mM-Mg\(^{2+}\), its effect seems to be less than that of Ca\(^{2+}\). The blocking effect of Ca\(^{2+}\) or Sr\(^{2+}\) is also likely to be membrane potential dependent since the rate of decline of the inward current at large negative membrane potentials tends to become faster as their concentration increases. This point was, however, not examined in detail in the present experiments.

As mentioned above, the inward current declines during the voltage pulse in the absence of Ba\(^{2+}\) at large negative membrane potentials. The decline found in the control solution is also due to the decrease in membrane conductance and not to
the change in driving force. This is similar to the decline of the current in the presence of Ba\textsuperscript{2+}. Since the control solution always contains 10 mM Ca\textsuperscript{2+} the decline of the current observed in the control solution, at least in part, represents the time course of the development of the voltage dependent blocking effect of Ca\textsuperscript{2+}. A small decline of the current remained, however, after removal of external Ca\textsuperscript{2+}. Some other ions such as Mg\textsuperscript{2+} may have a similar effect, or a ‘true’ inactivation of the membrane conductance may exist.

![Graph](image)

**Fig. 9.** A, membrane currents during voltage clamp at pH 7.0, 6.0, 5.5, 5.3 and 5.0 respectively. [K\textsuperscript{+}]\textsubscript{o} = 25 mM, holding potential \( V_0 \) = -53 mV. \( V-V_0 \) during the voltage pulse is listed for each current trace. Interrupted lines correspond to the level of zero current. B, normalized membrane current is plotted against pH. The three different symbols refer to measurements on three different preparations. The continuous curve is drawn according to eqn. (14).
**Blocking effect of H⁺ on the K current**

The effect of the pH of the external solution on the K current during the inward rectification was examined in 25 mm-K⁺. The zero-current membrane potential $V_0$ (-53 to -50 mV) showed no significant change over the pH range examined (pH 5–8). Membrane currents during voltage clamp pulses to various potentials from $V_0$ were recorded at different pH values (Fig. 9A). The membrane current for a given potential did not show any significant change until the pH was lowered below 6-0. Reducing the pH below this value resulted in a marked decrease in the current. In order to express the degree of suppression, the current at a given time from the onset of a voltage pulse to a given membrane potential was normalized by dividing by the current obtained at the same potential and time at pH 7-0. Although the amplitude of the current decreased at pH values below 6-0, the time course of the current was unaltered. Therefore, the normalized current is time-independent. The normalized current examined between $V - V_0 = -10$ and -50 mV is also independent of the membrane potential. However, the accuracy of the present measurements cannot exclude a slight voltage dependence (e.g. $\mu$ in eqn. (4) less than 0·2). The time- and voltage-independent action of H⁺ is in contrast to the blocking effect of Ba²⁺ ions discussed above.

The normalized current $I/I_{PH7.0}$ is plotted against pH in Fig. 9B. The continuous curve is drawn by assuming that three H⁺ ions must bind simultaneously to block each channel, i.e.

$$
\frac{I}{I_{PH7.0}} = \frac{K^3}{K^3 + [H^+]_0^3},
$$

(14)
in which $K$ is the apparent dissociation constant. The value of $pK$ is 5·3~5·4, or $K = 4~5 \mu M$. The current through the inward rectifier was greatly suppressed at pH 5·0. Fig. 9A shows that outward currents at positive membrane potentials obtained at pH 5·0 do not differ significantly from those obtained at pH 7·0. Since the outward current is likely to be carried by K⁺ ions, this indicates that the K channel for the outward rectification has a $pK$ lower than that of the K channel for the inward rectification.

**DISCUSSION**

The K conductance of the anomalous rectification depends on the potential displacement from $V_K$ rather than the membrane potential alone. Various models have been proposed to account for this property (Adrian, 1969; Armstrong, 1975). Recently, S. Ciani, S. Krasne, S. Miyazaki & S. Hagiwara (1978, submitted for publication) have proposed a model which describes this behaviour as resulting from the voltage-dependent orientation of charged molecules in the membrane followed by the voltage-independent binding of external K ions to the gating site of the oriented molecules. The relationship between the steady-state conductance and the membrane potential suggests that three charges move within the membrane to open the channel (Hagiwara & Takahashi, 1974). Therefore, three K⁺ ions are assumed to bind to the gating site in order to form a stable conductive channel. The concentration dependence of the H⁺ blockage suggests that three H⁺ ions simultaneously titrate the acidic groups of each channel; no significant membrane
potential dependence is found for the H\(^+\) effect. These properties can easily be explained from the above model by assuming that H\(^+\) ions titrate the gating sites which normally bind three K\(^+\) ions and thereby prevent the channel from becoming conductive. If the sites are close to the external surface of the membrane, the titration can be membrane potential independent.

The blocking effect of Ba\(^{2+}\) is membrane potential dependent. The steady-state blocking effect shows that one Ba\(^{2+}\) ion affects each channel. When the membrane potential is altered the blocking effect of Ba\(^{2+}\) changes to a new steady-state with a time course described by first-order kinetics. The membrane potential dependence of the Ba\(^{2+}\) blocking suggests that Ba\(^{2+}\) ions enter the membrane to bind to the channel at a point where the potential relative to the outside is \(\mu\) times the membrane potential. Our experimental results suggest that Ba\(^{2+}\) binds to conductive and non-conductive channels equally well. In the model by Ciani et al. (submitted) this may indicate that Ba\(^{2+}\) affects the oriented and unoriented molecule equally. Thus the site accessible to the external Ba\(^{2+}\) should be common to both the oriented and unoriented state. Although various possibilities can be considered to reconcile these observations with the model, they will not be discussed in this paper. The present experimental data can be explained by assuming that inactivation is independent of activation and in this regard it resembles the inactivation of the Na channel in the original Hodgkin–Huxley model (Hodgkin & Huxley, 1952). This, however, does not necessarily mean that the inactivation process introduced by Ba\(^{2+}\) is actually independent of the activation of the inward K channel. Coupling of the inactivation process to the activation process has been suggested for the Na channel of the squid axon (Bezanilla & Armstrong, 1977; Armstrong & Bezanilla, 1977).

A membrane-potential-dependent blockage of the channels has been found in a variety of preparations. Quaternary NH\(_4\) ions and their derivatives block the K channel of the outward rectification in the squid giant axon when applied internally and the effect increases as the membrane potential becomes more positive (Armstrong, 1969, 1971). Similar effects have been observed for internally applied Na\(^+\) and Cs\(^+\) ions (Bezanilla & Armstrong, 1972; French & Wells, 1977). According to Woodhull (1973) the blocking effect of external H\(^+\) ions on the Na channel of a myelinated nerve fibre decreases as the membrane potential becomes more positive. Our previous work shows that the K channel of the anomalous rectification is blocked also by Cs\(^+\) in a voltage-dependent manner (Hagiwara et al. 1976) but the behaviour of Cs\(^+\) blocking differs from that of Ba\(^{2+}\) blocking in several respects. In eqn. (4), \(\mu\) for Cs is greater than unity, being 1.4–1.5. Following a voltage step, the approach to steady-state blocking is substantially faster for Cs\(^+\) than for Ba\(^{2+}\); for example, the time constant for the Cs\(^+\) blocking is 50 msec whereas for Ba\(^{2+}\) is 2–3 sec for a comparable steady-state blocking. A third difference between Cs\(^{2+}\) and Ba\(^{2+}\) is found in the dependence of the apparent dissociation constant on the external K\(^+\) concentration. As already described, \(K(0)\) for Ba\(^{2+}\) in eqn. (4) is independent of [K\(^+\)]\(_{o}\). For Cs\(^{+}\), the blocking effect at a given membrane potential increases with increasing [K\(^+\)]\(_{o}\) when [Cs\(^{+}\)]\(_{o}\) is fixed, i.e. \(K(0)\) in eqn. (4) decreases with increasing [K\(^+\)]\(_{o}\).

Among monovalent cations examined, Cs\(^+\) (Hagiwara et al. 1976), Rb\(^+\) (Hagiwara & Takahashi, 1974; unpublished work by S. Hagiwara & S. Miyazaki, 1977) and Tl\(^+\)
(Hagiwara et al. 1977), show marked blocking effects on the K channel for the anomalous rectification. In contrast, no significant effect is found for Na⁺ or NH₄⁺ (Hagiwara & Takahashi, 1974). All three blocking cations have ionic radii greater than that of K (Robinson & Stokes, 1965). The ionic radius of Ba²⁺ is slightly greater than that of K and a strong blocking effect is found for Ba²⁺. Sr²⁺, Ca²⁺ and Mn²⁺ are smaller than K and they show slight blocking effects. The blocking sequence is Ba²⁺ > Sr²⁺ > Ca²⁺ > Mn²⁺, and coincides with the sequence of size. Thus, the crystal radius of the ion relative to that of K is probably one of the important factors determining the blocking efficacy. The size alone, however, cannot determine the action, since organic cations such as NH₄⁺, TEA⁺, choline⁺ and Tris⁺ show no significant blocking effect even though their sizes are either comparable to or greater than that of potassium.

The inward current at a large negative pulse potential declines even in the absence of Ba²⁺. The decline of the membrane current can be interpreted, at least in part, as the development of the voltage dependent blockage by Ca²⁺. A substantially more marked decline of the inward current is found for the anomalous rectification in frog skeletal muscle fibres (Almers, 1972a). The major cause of the decline in this case appears to be the change in the driving force as the result of depletion of K⁺ in the transverse tubular system. This effect is not seen in the starfish egg cell. Almers (1972b) has shown that a slight but significant decline of the current due to a voltage-dependent conductance decrease (‘inactivation’) is also found in the frog muscle fibre at large negative membrane potentials. The behaviour of the inactivation seen in the frog muscle fibre is very similar to that of the starfish egg observed in normal external medium. It is likely that the inactivation of the K current in both preparations may, at least in part, be due to the voltage-dependent blocking effect of some of the naturally occurring external cations such as Ca²⁺.

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REFERENCES


