# CONTROL OF THE HYPERPOLARIZATION-ACTIVATED CATION CURRENT BY EXTERNAL ANIONS IN RABBIT SINO-ATRIAL NODE CELLS

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## SUMMARY

1. Effects of varying concentrations of anions on the hyperpolarization-activated current ($I_f$) were studied in myocytes isolated from the rabbit sino-atrial node. Substituting $Cs^+$ for the intracellular $K^+$ clearly separated $I_f$ from the delayed rectifier $K^+$ current. Control properties, including gating kinetics and ion selectivity, similar to previous studies were obtained.

2. Substitution of extracellular $Cl^-$ with larger anions including isethionate, glutamate, acetate, and aspartate, reduced the amplitude of $I_f$ without changing the reversal potential. Substitution with small anions such as iodide or nitrate supported an intact $I_f$. These effects were reproduced in the excised outside-out patch conformation.

3. The conductance for $I_f$ was a saturating function of the extracellular $Cl^-$ concentration ($[Cl^-]_o$) with an equilibrium binding constant ($K_{\frac{1}{2}}$) of 11 mM and a slope factor of about 1 when substituted with large anions. Total removal of small anions completely abolished $I_f$.

4. The voltage-dependent gating of $I_f$ was not affected by changing ($[Cl^-]_o$), suggesting that $Cl^-$ modulates conductance properties of $I_f$.

5. The results indicate that $I_f$ conductance is unique in that it is dependent on an extracellular anion ($Cl^-$), yet it is carried exclusively by cations, $K^+$ and $Na^+$. These effects are independent of any measurable voltage-dependent gating parameters.

## INTRODUCTION

In various types of cells having a relatively low resting potential, hyperpolarization of the membrane induces an activation of an inward current ($I_f$ or $I_h$). The cell types include cardiac pacemaker cells (Seyama, 1979; Yanagihara & Irisawa, 1980; DiFrancesco, Ferroni, Mazzanti & Tromba, 1986), atrio-ventricular node cells (Noma, Irisawa, Kokobun, Kotake, Nishimura & Watanabe, 1980) and Purkinje cells (DiFrancesco, 1981 *a*, *b*, 1982), photoreceptors (Bader & Bertrand, 1984), and neurones in spinal sensory ganglia, and central nervous system (Mayer & Westbrook, 1983; McCormick & Pape, 1990). As a common characteristic among different cells,

$I_f$ shows a very slow time course of activation on hyperpolarization, relative to its quick deactivation on depolarization. The conductance of the $I_f$ channel is characterized by its multi-ionic nature. The amplitude of $I_f$ is increased by raising the extracellular $K^+$ concentration ($[K^+]_o$) and decreased by reducing $[Na^+]_o$. The measurement of the reversal potential suggests that the current is carried by both $K^+$ and $Na^+$ (DiFrancesco, 1981$a$). However, since the early examination of $I_f$ (Seyama, 1979), several studies have demonstrated that the current is also sensitive to alteration of $[Cl^-]_o$. Substitution of $Cl^-$ with large anions reduces the amplitude of $I_f$ (Yanagihara & Irisawa, 1980; Mayer & Westbrook, 1983; McCormick & Pape, 1990). The finding may suggest that $Cl^-$ also carries current through the $I_f$ channel under an assumption that the intracellular $Cl^-$ is depleted in the absence of external $Cl^-$ (Seyama, 1979). Alternatively, the finding may be explained by assuming a non-specific blocking action of impermeable anions (McCormick & Pape, 1990), or by assuming an activation of the channel by $Cl^-$ (present study).

In recent years several groups have refined the isolation technique of single sino-atrial (SA) node cells and are compiling more data on $I_f$ characteristics as well as its role in the pacemaker mechanism (DiFrancesco et al. 1986; Yatani & Brown, 1990; Denyer & Brown, 1990; van Ginneken & Giles, 1991). We have also developed an improved procedure for isolating SA node cells, in general agreement with these groups. We have undertaken a whole-cell voltage clamp analysis on the activation of $I_f$ and its dependence on anions by performing systematic ion-replacement experiments on isolated SA node cells.

## METHODS

### SA nodal cell isolation

Albino rabbits weighing 1–2 kg were anaesthetized with intravenous injection of sodium pentobarbitone (30–50 mg/kg). The heart was dissected out and mounted on a modified Langendorff perfusion system and perfused with Tyrode solution followed by a $Ca^{2+}$-free Tyrode solution for 3–5 min. After contractions ceased 300 ml of a $Ca^{2+}$-free Tyrode solution containing collagenase (Yakult, Tokyo, Japan; 0·13–0·03 mg/ml, depending on lot number) was perfused followed by 150 ml of $Ca^{2+}$-free Tyrode solution containing protease (Sigma, 0·1 mg/ml). The SA node region was then dissected out, trimmed, and cut into strips. A second $Ca^{2+}$-free collagenase digestion (0·6–0·4 mg/ml) was then performed for 10–30 min. The digested SA node tissue was then washed in a 'KB' solution (Isenberg & Klöckner, 1982) and dispersed by triturating with a fire-polished pipette. Dispersed cells were stored in 'KB' solution at 4 °C for up to 1 day. Cells used in this study were identified visually as long, spindle-shaped cells approximately 50–80 $\mu$m in length and 10–15 $\mu$m in width in normal Tyrode solution. These cells have faint striations and a prominent, centrally located nucleus (similar to type A in Denyer & Brown, 1990). Other cell types were generally larger, rod shaped, and striated, or of rounded shape. The present cell-isolation procedure and solutions used provide an $I_f$ that exhibits very little run-down in normal $Na^+$- or $K^+$-Tyrode solution.

### Solutions

Normal Tyrode solution contained (mM): NaCl, 140; KCl, 5·4; MgCl$_2$, 0·5; CaCl$_2$, 1·8; NaH$_2$PO$_4$, 0·33; glucose, 5·5 and HEPES, 5·0 (pH = 7·4). In recording $I_f$, BaCl$_2$ (2 mM) and NiCl$_2$ (1 mM) were also added to block other currents. In experiments which examined exchanging external anions, a sodium salt of aspartate, glutamate, acetate, isethionate, iodide, or nitrate was substituted for NaCl on an equimolar basis. In solutions requiring low $Cl^-$ concentrations, MgCl$_2$, NiCl$_2$ and BaCl$_2$ were omitted and calcium gluconate was used. Variations in $[Na^+]_o$ or $[K^+]_o$ were made by substituting them with Tris. All solutions were used at 37 °C.

The internal solution contained (mM): potassium aspartate or caesium aspartate, 110; KCl, 20; CaCl$_2$, 2·0; EGTA, 5·0; MgCl$_2$, 5·0; K$_2$ATP, 5·0, and HEPES, 5·0 (pH = 7·2). The free Ca$^{2+}$ is calculated to be 0·1 $\mu$M; 100 $\mu$M-GTP and 50 $\mu$M-cyclic AMP were included. In some instances 130 mM-CsCl was used as the major intracellular salt.

*Voltage clamp recording and analysis*

Whole-cell and isolated patch recordings were performed using the original methods of Hamill, Marty, Neher, Sakmann & Sigworth (1981). Currents were recorded with a patch clamp amplifier (List EPC-7, Darmstadt, Germany). The current, voltage, and trigger signals were stored on video tape using a PCM converter system (NF Electronic Instruments, RP-882, Tokyo, Japan) for subsequent computer analysis (NEC PC98 XL, Tokyo, Japan).

The liquid junction potentials of the aspartate-rich K$^+$ and Cs$^+$ internal solutions were $-11\cdot4\pm0\cdot3$ mV ($n = 10$) and $-12\cdot3\pm0\cdot7$ mV ($n = 7$), respectively, when in contact with the control Tyrode solution. The measurement of junction potential drifted by a few millivolts when the electrode pipette was kept in the Tyrode solution, most probably due to diffusion at the pipette tip. We do not know exactly how large the junction potential was between the pipette solution and the intracellular solution, although it should be small. Therefore, voltage recordings measured in the aspartate-rich internal solution were corrected by $-10$ mV for practical purposes. To minimize junction-potential error during Cl$^-$ variation a 3 M-KCl leakage electrode was used as a bath reference electrode. This reference system maintains a small, continuous leak of 3 M-KCl solution from a reservoir. The reference electrode was put downstream from the preparation. In other instances a large 3 M-KCl–agar bridge was used. Data are presented as means ± standard deviation.

## RESULTS

*Isolation of the hyperpolarization-activated current using the Cs$^+$-rich internal solution*

In nearly every spindle-shaped cell examined a large, inward current was activated on hyperpolarization negative to approximately $-70$ mV. After the initial break-in and voltage clamp, the current often decreased or increased to a steady-state level within minutes, presumably due to equilibration with the pipette solution, but the threshold potential for activation was not obviously changed. Rapid run-down of the current, as described in DiFrancesco *et al.* (1986), was not evident in normal Tyrode solution or in high-K$^+$ solutions, presumably due to a higher intracellular Ca$^{2+}$ of pCa 7 (Hagiwara & Irisawa, 1989) and inclusion of cyclic AMP and GTP (Yatani & Brown, 1990).

The activation of $I_f$ on hyperpolarization is usually overlapped with a time-dependent deactivation of the delayed rectifier K$^+$ current. To isolate $I_f$, the delayed rectifier K$^+$ current was blocked by adding 2 mM-Ba$^{2+}$ and 1 mM-Ni$^{2+}$ in the external solution, which also partially inhibited the time-independent background conductance. For complete suppression of the outward current of the delayed rectifier K$^+$ channel, Cs$^+$-rich internal solution was used. Figure 1$A$ shows a family of $I_f$ recordings in response to single hyperpolarizing steps. It is evident that the current trace at $-60$ mV shows no obvious time-dependent change and very slow activation of $I_f$ is visible at $-70$ mV. At stronger hyperpolarizations $I_f$ showed exponential activation at the onset of the pulse. With the Cs$^+$-rich solution the sigmoidal time course of the initial current was not significant.

The amplitude of the inward $I_f$ tail, which was recorded on terminating the hyperpolarizing pulse, increased with increasing conditional hyperpolarization and showed a trend of saturation. Voltage-dependent activation of $I_f$ was assessed by

measuring the tail current. Two different current parameters including integration of
the area of the tail current or measurement of the peak amplitude of tail current were
used. The values were normalized to the maximum value in each experiment. Data
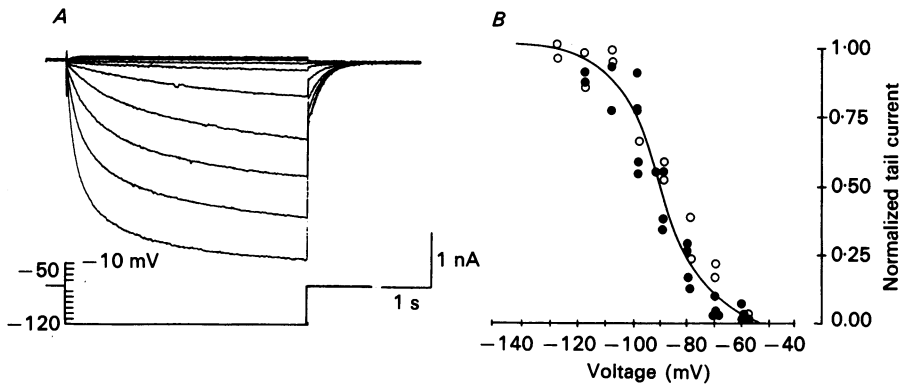obtained using the K$^+$-rich solution in the pipette are also shown for comparison. We



Fig. 1. Control current properties of $I_t$ recorded with the Cs$^+$-rich internal solution. $A$,
original current recordings are superimposed. The pulse protocol is shown in the lower
part: the holding potential was $-50$ mV and a 4·5 s pulse is applied in 10 mV increments.
Cs$^+$ pipette solution; Na$^+$ Tyrode solution with 2 mM-Ba$^{2+}$ and 1 mM-Ni$^{2+}$. $B$, voltage-
dependent activation of $I_t$ determined in Na$^+$ Tyrode solution using K$^+$ (◯) or Cs$^+$ (●)
pipette solution. The tail current values are normalized and plotted against membrane
potential for six experiments. The continuous line is a fit with eqn (1).

conclude that the activation curve is not affected by replacing internal K$^+$ with Cs$^+$.
All data points in Fig. 1$B$ were then fitted with the following Boltzmann relation:

$$K_{act} = \frac{1}{1 + \exp\{QF(V_m - V_{\frac{1}{2}})/RT\}},\qquad(1)$$

where $K_{act}$ is the activation parameter, $Q$ is valency of gating charge, $V_m$ is the
membrane potential, $V_{\frac{1}{2}}$ is the potential at half-maximum activation, and $F$, $R$ and
$T$ have their usual meanings in thermodynamics. Regardless of the major
intracellular cation or means of measuring the current amplitude, the activation
profile in Na$^+$ Tyrode solution displays a threshold near $-70$ mV, a $V_{\frac{1}{2}}$ of
$93·1 \pm 8·4$ mV, $Q = 3·03 \pm 0·55$ ($n = 6$; 4 intracellular Cs$^+$, 2 intracellular K$^+$). These
data are shown in Fig. 1$B$ with a representative fitted line.

The 'instantaneous' $I$–$V$ relations were measured from a double-pulse tail current
protocol as indicated in the insets of Fig. 2. Under physiological ionic conditions the
tail current reversed from inward to outward with depolarization at $-32·7 \pm 1·4$ mV
($n = 19$), which is slightly less negative than the intersection of the instantaneous $I$–$V$
curve. This is because the instantaneous $I$–$V$ relation is also determined by other
background conductances. The value of the reversal potential is in good agreement
with previous studies if corrected for the junction potential (DiFrancesco, 1981$b$;
van Ginneken & Giles, 1991). The value of the Na$^+$ conductance relative to the K$^+$
conductance (pNa/pK) is 0·27 according to the Goldman–Hodgkin–Katz equation.
With the Cs$^+$-rich solution the tail current was always inward and the instantaneous

*I–V* relation was a smooth curve (Fig. 2*B*). Thus, it is concluded that internal $Cs^+$ neither carries outward current, nor affects the inward flux through the $I_f$ channel. In the following experiments, data obtained with the $K^+$-rich solution were also used when the separation of potassium current ($I_K$) from $I_f$ was not critical in interpreting the experimental data.
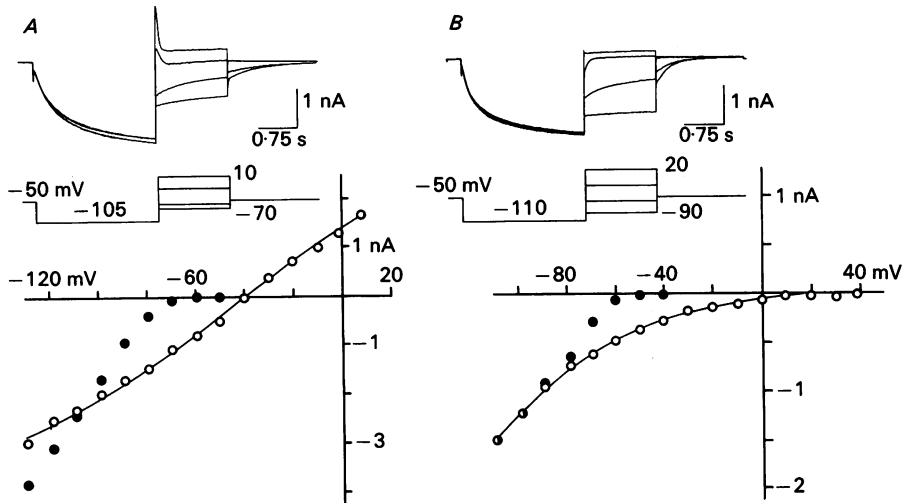


Fig. 2. Late current (●) and 'instantaneous' current–voltage relationships (○) recorded in potassium aspartate (*A*) and caesium aspartate pipette solutions (*B*) with the control $Na^+$ Tyrode solution. The late current was measured near the end of the hyperpolarizing pulse of the single-pulse protocol as shown in Fig. 1*A*. Current traces and the voltage protocols for the instantaneous *I–V* relationships are shown in the insets.

## Effects of anion replacement

Substitution of the majority of extracellular $Cl^-$ in $Na^+$ Tyrode solution by several organic anions was found to greatly reduce the amplitude of $I_f$, whereas substitution with small anions such as $I^-$ or $NO_3^-$ supported a full-amplitude $I_f$ with properties indistinguishable from control. Figure 3*A* shows representative records comparing the currents between control external $Cl^-$ and $NO_3^-$ (upper panel) or control $Cl^-$ and isethionate (lower). The amplitude of $I_f$ was reduced to about 30% of the control in the isethionate solution, but the time course of the current was not changed. The effects of different anions on the current amplitude were examined by normalizing the *I–V* relation referring to the amplitude of $I_f$ at $-120$ mV in the control. It was found, regardless of species, that all the organic anions tested reduced $I_f$ to nearly the same extent, and that the current amplitude was almost identical with $Cl^-$, $I^-$, and $NO_3^-$ (Fig. 3*B*). The amplitude of the current in the organic anion solutions is about 30% of the control over the potential range $-120$ to $-90$ mV, indicating no obvious voltage dependence of the effect of $Cl^-$ replacement. These findings are difficult to explain by assuming a blocking action of organic anions (McCormick & Pape, 1990), since the molecular structure of these ions is different.

No measurable $Cl^-$ permeability was evident from experiments of replacing external $Cl^-$. The reversal potential of $I_f$ was measured by plotting the instantaneous
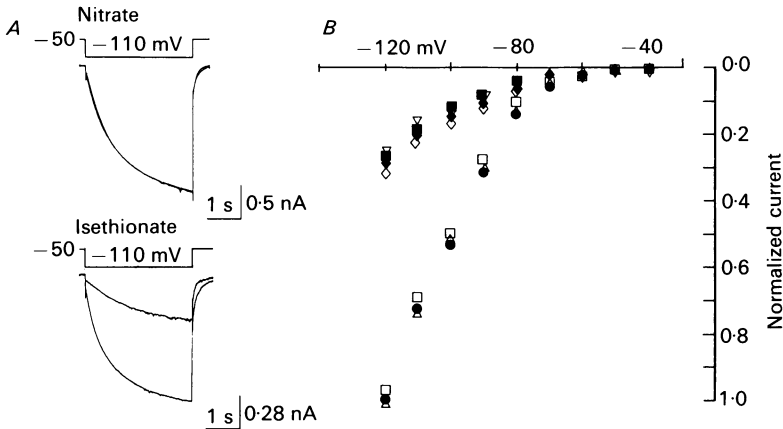
Fig. 3. A, current traces at −110 mV before and after substituting $NO_3^-$ (upper panel) and isethionate (lower panel) for Cl⁻ in Tyrode solution are superimposed. B, late current–voltage relationships in Na⁺ Tyrode solution using different extracellular anions, aspartate (■), glutamate (▽), acetate (◆), isethionate (◇), Cl⁻ (△), I⁻ (□), or $NO_3^-$ (●). The Cl⁻ from $CaCl_2$, $MgCl_2$, $NiCl_2$ and $BaCl_2$ remains in the extracellular solution (10·6 mM). Currents were normalized to the peak inward current at the maximum hyperpolarized potential. The instantaneous current jumps at the pulse onset were assumed to be due to leak current systems and were subtracted.
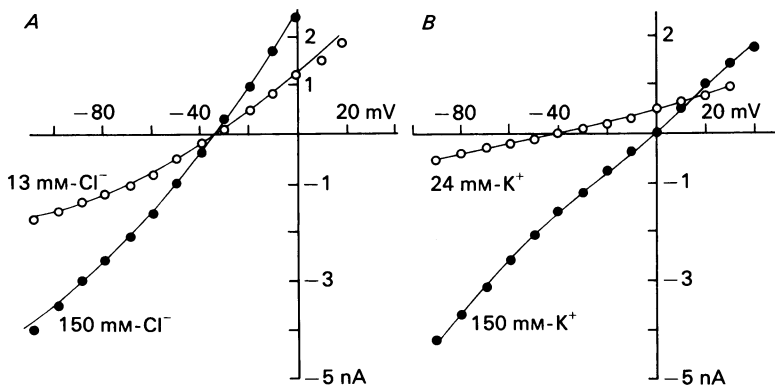


Fig. 4. The instantaneous I–V relations in K⁺ Tyrode solution containing 150 (●) and 13 (○) mM-Cl⁻ aspartate substitution in A, and those obtained at a constant $[Cl^-]_o$ of 150 mM in 24 mM $[K^+]_o$ (○; Tris substitution) and 150 mM $[K^+]_o$ solutions (●) in B. Pulse protocol is essentially the same as shown in Fig. 2.

I–V relation before and after the reduction of $[Cl^-]_o$ from 150 to 13 mM (aspartate replacement) in 150 mM-external Na⁺ (Fig. 4A). The slope of the instantaneous I–V curve is reduced at all potentials at low $[Cl^-]_o$. Although the Cl⁻ reversal potential was expected to change by about 64 mV from −39 mV in the control to +25 mV in 13 mM-Cl⁻, the reversal potential remained constant at around −33 mV, which is determined by both Na⁺ and K⁺. This finding may also indicate that the ion

selectivity, $pNa/pK$, is not affected by lowering $[Cl^-]_o$. When $[K^+]_o$ was changed from 150 to 24 mM at a constant $[Cl^-]_o$ in the $Na^+$-free solution (Tris-HCl replacement), the reversal potential shifted as expected from a change in the $K^+$ equilibrium
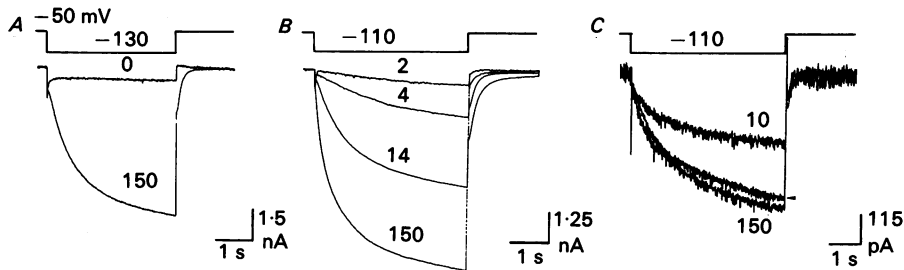


Fig. 5. Effects of varying $[Cl^-]_o$ in the whole cell ($A$ and $B$) and the outside-out patch ($C$) in $K^+$ Tyrode solution. $A$, current traces obtained in control 150 mM-$Cl^-$ and after total replacement of $Cl^-$ with aspartate are superimposed. The pulse protocol is indicated above the current. $B$, currents recorded at $[Cl^-]_o$ of 2, 4, 14 and 150 mM are superimposed. $C$, the control records at 150 mM $[Cl^-]_o$ obtained before (150, lower trace) and after (arrow-head) the application of 10 mM-$Cl^-$ are superimposed with the test run (10).

potential (Fig. 4$B$). Thus we conclude that $Cl^-$ is not permeating the $I_f$ channel, which is a cation channel.

*Dose-dependent potentiation of $I_f$ by small anions*

An activating action by $Cl^-$, rather than $I_f$ channel block by organic anions, was strongly supported by recording $I_f$ at very low $[Cl^-]_o$. The $K^+$ Tyrode solution was used to obtain the maximum current in these experiments. On complete substitution of $Cl^-$ with aspartate virtually no $I_f$ was recorded, even with large voltage steps (Fig. 5$A$). Addition of only 2, 4, or 14 mM-$Cl^-$ to the external solution induced an $I_f$ of progressively larger amplitudes during hyperpolarizing pulses (Fig. 5$B$). In these solutions changes in the concentration of organic anions is less than 10% of the control concentration. It is difficult to assume that decreasing the concentration of the organic anion from 148 to 136 mM progressively released the channel block.

The cell-free patch recording of channel current may be free from complications arising from cell-induced mechanisms, such as changes in cell volume or intracellular ionic concentrations and pH, stimulated by the $Cl^-$ replacement. Recording of $I_f$ from a cell-free patch has already been reported, initially by Yatani & Brown (1990). Therefore, the protocol was also performed using isolated outside-out patches. Although the $I_f$ in this conformation underwent run-down, currents were rapidly reduced in 10 mM-$Cl^-$ and recovered in control $Cl^-$ solution (Fig. 5$C$). Also, experiments were conducted with $Cl^-$ replacing aspartate as the major intracellular anion ($[Cl^-]_i = 140$ *vs.* 24 mM in the normal control) with no change in the effect.

The findings described above strongly suggest that the external presence of small anions is essential for activation of the $I_f$ channel by hyperpolarization. Dose dependence of this effect was quantified by measuring the steady-state current at
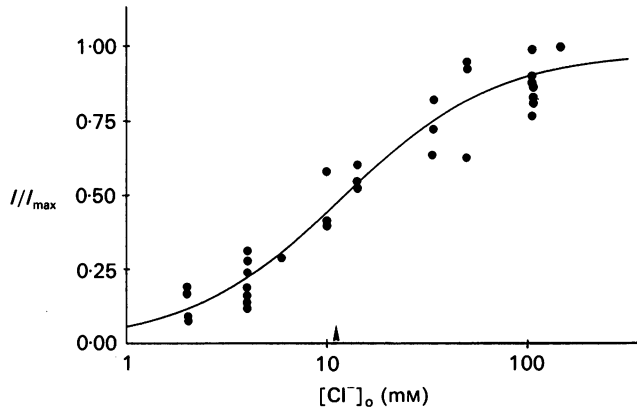
Fig. 6. Dose–response relationship for peak $I_f$ vs. $[Cl^-]_o$. The amplitudes of $I_f$ in each experiment ($n = 32$) are normalized to the peak current obtained at $-120$ mV in control 150 mM-KCl Tyrode solution which preceded the reduced $Cl^-$ application. The continuous line is the fit to the Hill equation. The arrow-head points to the $K_{\frac{1}{2}}$ concentration.
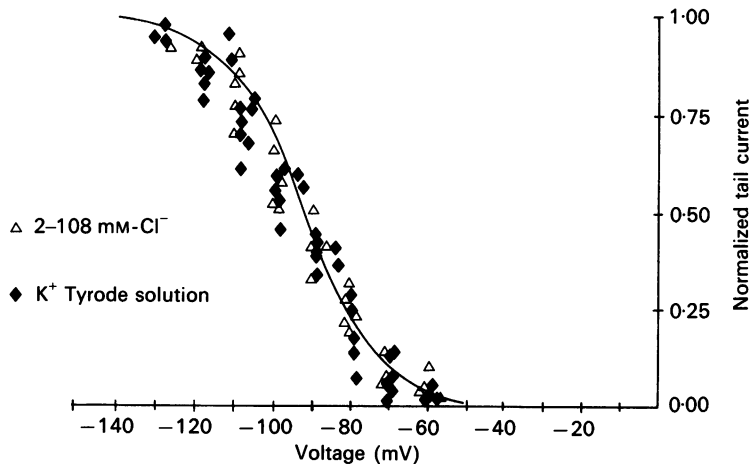


Fig. 7. Voltage-dependent activation in various $[Cl^-]_o$ ($\triangle$) compared to those obtained in the 150 mM $[Cl^-]_o$ in $K^+$ Tyrode solution ($\blacklozenge$). Data in each experiment are normalized to the peak tail current. Continuous line, fitting eqn (1), is for the control $K^+$ data.

a single, large, hyperpolarizing test potential ($-120$ mV) over a wide range of $[Cl^-]_o$ (substituting with glutamate or aspartate) and a dose–response relation was compiled ($n = 32$). The concentration dependence was fitted with a single-site binding relation with an equilibrium binding constant ($K_{\frac{1}{2}}$) of 11·5 mM and a Hill coefficient of 1·13 (Fig. 6).

*Mechanisms of $I_f$ depression in low-$Cl^-$ solution*

The reduction in the amplitude of $I_f$ might be caused by a change in the voltage-operated gating kinetics, or by a reduction in the single-channel conductance in the low-$Cl^-$ solution. We failed to record single-channel events in the cell-attached patch recording or in the excised outside-out patch, most probably due to extremely small

single-channel conductance (DiFrancesco, 1986). However, we could test the gating
kinetics at low [Cl⁻]$_o$. Figure 7 compares the quasi-steady-state activation–voltage
relationship between $I_f$ in the lower [Cl⁻]$_o$ solutions and that measured at
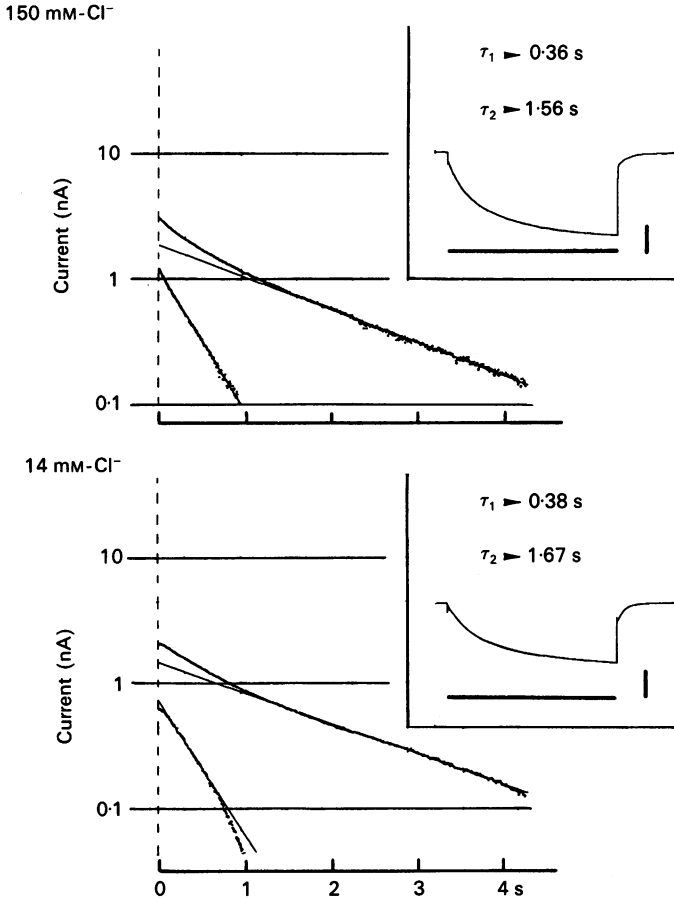140 mM [Cl⁻]$_o$ in the K⁺ Tyrode solution. In six experiments, there was little effect



Fig. 8. Semilog plots of the time-dependent activation of $I_f$. The upper panel shows those
for control 150 mM [Cl⁻]$_o$ in K⁺ Tyrode solution, and the lower panel at 14 mM [Cl⁻]$_o$
(aspartate substitution). The total time-dependent current (upper point plots) and the
subtracted fast exponential component (lower plots) are shown. The theoretical curve, a
sum of the slow and fast components, was superimposed on the original current in the
insets, although the good fit made the two traces indistinguishable. The horizontal bar in
the inset indicates the duration of the test pulse ($-100$ mV) of 5 s, and the vertical bar
1 nA. See text for detail.

on the voltage-dependent activation parameters over this concentration range. The
$V_\frac{1}{2}$ in low Cl⁻ was $94\cdot4 \pm 3\cdot7$ mV and $Q$ was $2\cdot53 \pm 0\cdot56$.

   The rate of $I_f$ activation was measured by plotting the current on a logarithmic
scale. In Fig. 8, the ordinate indicates the current amplitude measured in reference
to an assumed steady-state level. Since the current did not reach a steady level

during the pulse, it was necessary to assume a steady-state level. To determine an appropriate value, the reference level was arbitrarily shifted until the semi-logarithmic plot became linear near the end of the pulse as shown in the figure. By calculating the regression line over the late linear portion, the slow component was determined first. Then this slow component was subtracted from the original current, and the difference was replotted to measure the fast component. The sum of these two exponential components almost perfectly fitted the original current record as shown in the inset of Fig. 8. It should be noted that at small hyperpolarizations a single exponential often gave a good fit to the currents. On reduction of the current in 14 mM $[Cl^-]_o$ the current kinetics are nearly unaffected with rate constants ($\tau$) of 0·38 and 1·67 as compared with the control values of 0·36 and 1·56 s. Essentially the same results were obtained in three experiments.

## DISCUSSION

The results of this paper address the question of how substitution of extracellular $Cl^-$ by large anions reduces the size of $I_f$. This effect of $Cl^-$ substitution has been described, initially by Seyama (1979), followed by Yanagihara & Irisawa (1980) in cardiac SA node. More recently, a similar phenomenon has been described for the very similar $I_f$ in spinal sensory ganglion neurones (Mayer & Westbrook, 1983) and in thalamic relay neurones (McCormick & Pape, 1990). These latter reports suggested a non-specific blocking activity for the large anions. On further $Cl^-$ removal or on addition of only a few millimoles of $Cl^-$ in the presence of high concentrations of organic anions (Fig. 3), it is clear that $I_f$ is directly dependent on small anions in the extra-cellular solution. Anions of < 0·25 nm seem to be able to support a normal current while larger, organic anions allow none. The response is a membrane-delimited one in that it is sustained in an excised-patch conformation. Also, intracellular $Cl^-$ depletion was avoided in experiments containing high $Cl^-$ in the pipette solution.

Dual involvement of anions and cations in a single-conductance system is not a novel result, having been described for anion permeability of gramicidin channels (Neher, 1975) and for cation permeability of an anion channel in hippocampal neurones (Franciolini & Nonner, 1987). However, each of these systems is found to have an experimentally measurable permeability of both anion and cation. We can find no measurable $Cl^-$ permeability in the $I_f$ system. No shifts in reversal potential are found after large changes in $[Cl^-]_o$. Furthermore, removing $Cl^-$ from the bath solution for the cardiac and neuronal preparations would lead to an increase of inward current rather than a decrease, if anion permeation is a factor.

A model for binding of impermeant anions has been proposed to interpret changes in thallous ion conductance in the gramicidin channel (Eisenman, Sandbloom & Neher, 1978). The model proposes that small anions perform a screening role for cations bound at external sites of a multi-ion pore. The effects on gramicidin channel conductance are quite small in comparison with the present result. The cardiac $I_f$ conductance was totally eliminated by completely omitting external $Cl^-$. We postulate that such a type of positive charge screening, whether it be a fixed charge at a recessed site on the protein or a bound cation is a necessary step in $I_f$ channel permeation by cations, such as $Na^+$ and $K^+$. Access by the large organic anions to the screening sites may be sterically blocked by a small tunnel structure of the channel

protein. The voltage-independent nature of this $Cl^-$ effect suggests that these interactions should be out of the membrane potential field in the case of the cardiac $I_f$ channel. This view is consistent with the finding that the voltage-operated gating properties were not modified by varying the $Cl^-$ concentration. The voltage-dependent gating should depend on a molecular structure within the membrane potential field. The fact that voltage-dependent activation and $I_f$ kinetics are unaffected by $Cl^-$ variation suggests that the manner by which $Cl^-$ exerts its effect is by varying channel conductance. While a change in either unit conductance or open probability could produce such a macroscopic result, it seems likely that such a large change in open probability would also be reflected in either gating kinetics or activation of $I_f$, as it is in other ion-gated channels (for example the $Ca^{2+}$-activated $K^+$ channel, Barrett, Magleby, & Pallotta, 1982). Although a novel $Cl^-$ binding scheme which produces a functional channel in an all-or-none manner, without subsequent effects on channel properties, could be argued, we reason that a $Cl^-$ effect on $I_f$ channel permeation is the most likely interpretation. Direct single-channel measurement of these parameters is required to test these hypotheses.

The present study succeeded in separating the activation of $I_f$ from the deactivation of the $I_K$ by using $Cs^+$ in the internal solution. It should be noted that the deactivation of $I_K$ causes a time-dependent change of the whole-cell current in the same direction as the activation of $I_f$ over the potential range positive to the $K^+$ equilibrium potential. In the present study, the outward current through the delayed rectifier $K^+$ channel was suppressed by replacing the intracellular $K^+$ with $Cs^+$, but the inward current through $I_f$ channel was not blocked by the $Cs^+$. The finding is in contrast to the block of the $I_f$ channel with $Cs^+$ from the external side (DiFrancesco, 1982; Noma, Morad & Irisawa, 1983). It may be speculated that the $I_f$ channel does not allow access of $Cs^+$ from the internal mouth of the channel. The asymmetrical effect of $Cs^+$ across the membrane is well established for the inward rectifier $K^+$ channel (see, for example, Matsuda & Noma, 1984). If the ion selectivity of the $I_f$ channel in the presence of 5·4 mm-$K^+$ in the external solution is determined by the Goldman–Hodgkin–Katz equation using the reversal potential of about $-33$ mV in the present study, the pNa/pK ratio is only 0·27. Thus, the $I_f$ channel is also relatively selective for $K^+$.

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