THE EFFECT OF Divalent Cations
ON I\textsubscript{K(f)}, A TRANSIENT OUTWARD POTASSIUM
CURRENT EXPRESSED IN MELANOTROPHS
OF THE RAT PITUITARY GLAND

by

JANA-LEA DAVIDSON
B.Sc., University of British Columbia, 1985

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
(Department of Physiology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
September 1992
© Jana-Lea Davidson
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature)

Department of **Physiology**

The University of British Columbia
Vancouver, Canada

Date **SEPT. 30, 1992**
ABSTRACT

Divalent cations are known to exert a charge screening effect on voltage-gated ion channels either through non-specific interactions with fixed negative charges on the cell membrane or via binding to negatively charged sites on or electrically close to the channel forming protein. In some instances, divalent cations bind directly to the gating apparatus of voltage-sensitive sodium and potassium channels thereby stabilizing the channels in a closed conformation.

Most of the investigations into the effects of divalent cations on voltage-gated ion channels have concentrated on the Na\(^+\) and delayed rectifier type K\(^+\) channels. Although, there has been a recent explosion of information regarding the molecular structure of the transient outward potassium channel, few investigators have examined the actions of divalent cations on the behaviour of the transient outward potassium current (TOC).

A transient outward potassium current, (I\(_K(f)\)), has been characterized in melanotrophs, the major cell type found in the pars intermedia of the pituitary gland in rats (Kehl, 1989). I\(_K(f)\) activates and inactivates rapidly. Cd\(^{2+}\) (5 mM) reduced the peak amplitude of I\(_K(f)\) and increased the 50% rise time of this current (Kehl, 1989). The present study elaborated on these observations and examined the effects of varying the extracellular concentrations of Cd\(^{2+}\), Zn\(^{2+}\), Ca\(^{2+}\) and Mg\(^{2+}\) on the behaviour of I\(_K(f)\).

Acutely dissociated melanotrophs were obtained from male Wistar rats and whole-cell currents were recorded, using conventional patch clamp techniques, from cells maintained in culture for 1-14 hrs.

Divalent cations shift the activation and inactivation curves and the gating kinetics of I\(_K(f)\) right-ward along the voltage axis. The cations tested varied in their ability to shift the potential-sensitive parameters of I\(_K(f)\) and ranked in the following order: Zn\(^{2+}\) \(>\) Cd\(^{2+}\) \(>\) Ca\(^{2+}\) \(>\) Mg\(^{2+}\), in good agreement with previous observations of their effect on sodium channels.
The mean control half-activation potential was -13.6 mV with a slope-factor of +12.8 mV (n=55) and the mean control half-inactivation potential was -54.7 mV with a slope-factor of -4.4 mV (n=50). The relationships between the shift of the half-activation potential and the divalent cation concentration indicated that the $K_M$'s for the half-maximal shift of the activation curve were 221 µM (Cd$^{2+}$), 92 µM (Zn$^{2+}$) and 3.4 mM (Ca$^{2+}$) and the maximal shifts of the activation curve were, respectively, +28 mV, +34 mV and +15.6 mV. Mg$^{2+}$ was far less potent than any of the other divalent cations examined. Shifts of the inactivation curve were equal to the shifts of the activation curve at each divalent cation concentration tested. The slope-factors of the activation and inactivation curves were not altered by the application of divalent cations. Removal of Ca$^{2+}$ from the external media significantly increased the slope-factor for the activation curve. That is, zero Ca$^{2+}$ resulted in a decrease in the equivalent charge transferred during the activation gating process.

The prediction central to non-specific charge screening is that all divalent cations will be equally effective. The results reported here show that this is not the case for $I_K(f)$. It is proposed that a specific binding site for divalent cations exists on or electrically close to the channel protein. Divalent cations also slowed the rise time of $I_K(f)$ suggesting that they might stabilize the channel conducting this current in the closed conformation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Voltage-Gated Ion Channels And Electrical Signals In Excitable Cells</td>
<td>1</td>
</tr>
<tr>
<td>Negative Surface Charges Exist On Cell Membranes</td>
<td>2</td>
</tr>
<tr>
<td>Membrane Surface Potential Can Be Described Mathematically</td>
<td>4</td>
</tr>
<tr>
<td>Different Species Of Divalent Cations Are Not Equally Effective In</td>
<td>4</td>
</tr>
<tr>
<td>Shifting The Voltage-Sensitive Parameters Of Voltage-Gated Ion Channels</td>
<td>4</td>
</tr>
<tr>
<td>Some Species Of Divalent Cations Block Ion Channels In A Voltage-Dependent Manner</td>
<td>6</td>
</tr>
<tr>
<td>Molecular Characterization Of Voltage-Gated Ion Channels</td>
<td>7</td>
</tr>
<tr>
<td>Is The Behaviour Of The Transient Outward Potassium Channel Influenced By Divalent Cations?</td>
<td>8</td>
</tr>
<tr>
<td>Melanotrophs Possess A Transient Outward Potassium Current</td>
<td>9</td>
</tr>
<tr>
<td>Experimental Rationale</td>
<td>10</td>
</tr>
<tr>
<td>METHODS</td>
<td>11</td>
</tr>
<tr>
<td>1. Preparation Of Acutely Dissociated Melanotrophs</td>
<td>11</td>
</tr>
<tr>
<td>2. Electrophysiology</td>
<td>12</td>
</tr>
<tr>
<td>3. Data Acquisition and Analysis</td>
<td>13</td>
</tr>
<tr>
<td>4. Recording Solutions</td>
<td>14</td>
</tr>
</tbody>
</table>
RESULTS..............................................................................................................................18

SECTION I: GENERAL PROPERTIES OF I_K(f).............................................................................18

1. Voltage- and Time-Dependence Of Activation................................................................18
2. Voltage- and Time-Dependence Of Inactivation.................................................................19
4. Reversal Potential...............................................................................................................22

SECTION II: THE EFFECTS OF DIVALENT CATIONS ON I_K(f).................................................27

1. The Effects Of Transition Metal Ions On The Gating Of I_K(f)...........................................27
   1.1 Cadmium.........................................................................................................................27
      1.11 Cd^{2+} Reduces The Peak Amplitude Of I_K(f).........................................................27
      1.12 Cd^{2+} Causes A Right-Ward Shift Of The Activation Curve.................................33
      1.13 Concentration Dependence For Cd^{2+}-Induced Shifts Of V'.................................34
      1.14 Cd^{2+} Increases The 50% Rise Time (t_{50\text{act}}) Of I_K(f).....................................34
      1.15 Cd^{2+} Causes A Right-Ward Shift Of The Steady-State Inactivation (h_{e}) Curve........................................................................................................................................37
      1.16 Cd^{2+} Increases The Time To Half-Inactivation (t_{50\text{inact}})....................................38
   1.2 Zinc................................................................................................................................29
      1.21 Zn^{2+} Reduces The Peak Amplitude Of I_K(f).........................................................39
      1.22 Zn^{2+} Causes A Right-Ward Shift Of The Activation Curve....................................44
      1.23 Concentration Dependence For Zn^{2+}-Induced Shifts Of V'...................................47
      1.24 Zn^{2+} Increases The 50% Rise Time Of I_K(f)..........................................................47
      1.25 Zn^{2+} Causes A Right-Ward Shift Of The Steady-State Inactivation Curve...............47
      1.26 Zn^{2+} Increases The Time To Half-Inactivation.........................................................50
2. The Effects Of Alkaline Earth Metal Ions On $I_K(f)$

2.1 Calcium

2.11 Changes in $[Ca^{2+}]_o$ Reduce $I_K(f)$

2.12 Changes in $[Ca^{2+}]_o$ Shift $V'$ For The Activation Curve

2.13 Concentration Dependence For $Ca^{2+}$-Induced Shifts Of $V'$

2.14 Raising $[Ca^{2+}]_o$ Increases $t_{\text{act}}$ For $I_K(f)$

2.15 Changes In $[Ca^{2+}]_o$ Shift $V'$ For The Inactivation Curve

2.16 Influence Of Changing $[Ca^{2+}]_o$ On $t_{\text{inact}}$ For $I_K(f)$

2.2 Magnesium

2.21 Voltage- and Time-Dependence Of Activation For $I_K(f)$ In The Presence Of $Mg^{2+}$

2.22 $Mg^{2+}$ Shifts $V'$ For Steady-State Inactivation

DISCUSSION

Divalent Cations Exert A Charge Screening Effect On The Channel Conducting $I_K(f)$

Divalent Cations Stabilize The Closed Conformation Of The Channel Conducting $I_K(f)$

Speculation On The Possible Characteristics Of The Binding Site

High $[Zn^{2+}]_o$ And $[Cd^{2+}]_o$ Reduce $G_{\text{max}}$

Is $Ca^{2+}$ A Necessary Co-factor For $I_K(f)$?

The Activation And Inactivation Of $I_K(f)$ Appear To Be Coupled

Physico-Chemical Properties Of The Divalent Cations

Summary and Future Directions

Concluding Remarks

BIBLIOGRAPHY
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The current-voltage relation and steady-state inactivation of $I_K(f)$ under control conditions.</td>
</tr>
<tr>
<td>2</td>
<td>The time constants for the inactivation of $I_K(f)$.</td>
</tr>
<tr>
<td>3</td>
<td>The reversal potential for $I_K(f)$ as determined from tail currents.</td>
</tr>
<tr>
<td>4</td>
<td>The concentration-dependent effects of Cd$^{2+}$ on $I_K(f)$.</td>
</tr>
<tr>
<td>5</td>
<td>The effects of 500 $\mu$M and 5 mM Cd$^{2+}$ on the voltage-dependence of activation and inactivation for $I_K(f)$.</td>
</tr>
<tr>
<td>6</td>
<td>Concentration-dependent effects of Cd$^{2+}$ on the shift of the half-activation potential.</td>
</tr>
<tr>
<td>7</td>
<td>The time-dependence of activation and inactivation in the presence of 500 $\mu$M and 5 mM Cd$^{2+}$</td>
</tr>
<tr>
<td>8</td>
<td>Concentration-dependent effects of Zn$^{2+}$ on the current-voltage relation of $I_K(f)$.</td>
</tr>
<tr>
<td>9</td>
<td>Control and treated activation and inactivation curves in Zn$^{2+}$ concentrations of 62.5 $\mu$M, 125 $\mu$M, 250 $\mu$M, and 500 $\mu$M.</td>
</tr>
<tr>
<td>10</td>
<td>The concentration dependence of the Zn$^{2+}$-induced shift of the half-activation potential for $I_K(f)$.</td>
</tr>
<tr>
<td>11</td>
<td>The time-dependence of activation and inactivation in the presence of 500 $\mu$M Zn$^{2+}$.</td>
</tr>
<tr>
<td>12</td>
<td>Current-voltage relations for normalized $I_K(f)$ in response to reduced or increased concentrations of external Ca$^{2+}$.</td>
</tr>
<tr>
<td>13</td>
<td>The effect of changes of $[\text{Ca}^{2+}]_o$ on the activation and steady-state inactivation curves for $I_K(f)$.</td>
</tr>
<tr>
<td>14</td>
<td>Concentration-response for the effects of Ca$^{2+}$ on the shift of the half-activation potential ($V'$).</td>
</tr>
<tr>
<td>15</td>
<td>The effect of increased $[\text{Ca}^{2+}]_o$ on the activation and inactivation kinetics of $I_K(f)$.</td>
</tr>
</tbody>
</table>
16 The effects of 10 mM (A) and 40 mM (B) Mg$^{2+}$ on the activation and inactivation curves for $I_K(f)$ ................................................................. 72

17 The time-dependence of activation in the presence of 40 mM Mg$^{2+}$ .......... 75
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Composition of external solutions</td>
</tr>
<tr>
<td>2</td>
<td>The values of ( V' ) and ( k ) calculated for the activation and inactivation of ( I_{K}(f) ) in the presence of ( \text{Cd}^{2+} )</td>
</tr>
<tr>
<td>3</td>
<td>The values of ( V' ) and ( k ) calculated for the activation and inactivation of ( I_{K}(f) ) in the presence of ( \text{Zn}^{2+} )</td>
</tr>
<tr>
<td>4</td>
<td>The values of ( V' ) and ( k ) calculated for the activation and inactivation of ( I_{K}(f) ) in the presence and absence of ( \text{Ca}^{2+} )</td>
</tr>
<tr>
<td>5</td>
<td>The values of ( V' ) and ( k ) calculated for the activation and inactivation of ( I_{K}(f) ) in the presence of ( \text{Mg}^{2+} )</td>
</tr>
<tr>
<td>6</td>
<td>Shifts of the activation and inactivation curves of ( I_{K}(f) ) caused by altering the external divalent cation concentration</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Dr. Steven Kehl for allowing me to work with him, for his invaluable advice and input during the course of this project and for providing me with a student fellowship. In particular, I would like to thank Dr. Kehl for sharing with me his dedication to his craft and for teaching me by his fine example how I should approach not only science and research but learning.

My sincere thanks to Dr. Peter Vaughan for always making time for my questions, for the hours spent at the chalk board and for his encouragement and input throughout my thesis project. I would also like to thank Dr. Vaughan for encouraging me to undertake this Master's degree.

Thank-you as well to Dr. David Mathers for Chairing my thesis committee, for keeping all the necessary paper work flowing, for his thorough review of my thesis and for his helpful comments and observations thereafter.

A big thank-you to Dr. Raymond Pederson for his endless efforts as the Graduate Student Advisor which made the trek through graduate studies in Physiology such a wonderful experience. Memories of the Mayne Island retreats will always make me smile!

A special thank-you to CA, Christine, and Jim for your friendship and encouragement and for agreeing to adjourn the Saturday morning meetings at Pauls! A very special thank-you to Jim Potts for rescuing me from a sea of numbers and helping with the statistical analysis.

Thanks to the rest of the graduate students and staff in Physiology for making my stay seem too short, to Khaled for our wonderful discussions and to Eric for all his musical talents and his bad jokes!

I would like to thank Clara for her technical assistance and Monica for her assistance in the preparation of the figures for this thesis.

To my family, thank-you hardly seems enough, your love and support has always allowed me to take on new challenges.

Scoobie, I couldn't have done it without you...thanks isn't enough.
INTRODUCTION

Voltage-Gated Ion Channels And Electrical Signals In Excitable Cells

Voltage-sensitive ion channels amplify electrical signals and allow them to propagate along the membrane from one region of an excitable cell to another. These channels, which are activated by local changes in membrane potential, are responsible for the dramatic increase in the permeability of excitable membranes to Na\(^+\) and K\(^+\) during the firing of an action potential.

Hodgkin and Huxley (1952), who first described these permeability changes in squid axon, proposed the existence of channels selective for Na\(^+\) and K\(^+\) and hypothesized that independent gating parameters responding to changes in membrane potential controlled the activation and inactivation of the Na\(^+\) channel and the activation of the K\(^+\) channel. Of great importance to the field of electrophysiology was the testable mathematical model they developed which predicted the behaviour of these gating parameters and mimicked the Na\(^+\) and K\(^+\) currents they observed (Hodgkin & Huxley, 1952).

Hodgkin and Huxley (1952) also described the driving forces which would push ions through these open channels. At rest the membranes of excitable cells are far more permeable to K\(^+\) ions than to Na\(^+\) ions. This reduces the amount of Na\(^+\) crossing the membrane and reduces the metabolic energy required to keep the intracellular concentration of Na\(^+\) low. On the other hand, the intracellular concentration of K\(^+\) is kept high and the resting membrane potential usually lies close to \(E_K\), the equilibrium potential for K\(^+\). Thus, the potential of the intracellular compartment is negative with respect to the extracellular compartment (Hodgkin & Huxley, 1952). When a channel opens the driving force provided by this electrochemical gradient allows the movement of ions across the cell membrane (reviewed in Hille, 1984).

Since Hodgkin and Huxley's definitive work studies of excitable cells from many other species and tissues have led to the discovery of voltage-gated Ca\(^{2+}\) and Cl\(^-\) channels as well
as other Na\(^+\) and K\(^+\) channels. The use of more sophisticated techniques for analyzing the behaviour of these channels has increased the knowledge of how they behave which is reflected in commensurately more complicated mathematical models (reviewed in Hille, 1984).

Excitable cells are usually studied in environments created by the investigator and conclusions regarding the manner in which these cells function in situ are often drawn from these experiments. Voltage-gated ion channels play an important role in signal propagation in excitable cells. Therefore, understanding how factors, such as the ionic composition of the recording solutions, influence the conductance through these channels or the voltage-sensitive gating of these channels becomes very important. Conversely, the manner in which these channels respond to changes in their environment provides some insight into structure/function relationships.

**Negative Surface Charges Exist On Cell Membranes**

Intracellular or extracellular changes in divalent cation concentration (Frankenhaeuser & Hodgkin, 1957; McLaughlin et al., 1971; Hille et al., 1975), ionic strength (Chandler et al., 1965; Mozhayeva and Naumov, 1972), or pH (Hille, 1968; Mozhayeva and Naumov, 1972; Woodhull, 1973) shift the potential-sensitive parameters of voltage-dependent ion channels along the voltage axis. Several models have been proposed to account for the voltage shifts induced by these manipulations including, screening of fixed negative surface charges on the membrane, specific binding of cations to negative surface charges and combinations of both (Frankenhaeuser and Hodgkin, 1957; Gilbert and Ehrenstein, 1969; McLaughlin et al., 1971; D'Arrigo, 1978; Hille, 1968; Hille et al., 1975). These experimental conditions appear to alter the membrane potential surrounding the voltage sensor of the channel without altering the bulk potential between the intracellular and extracellular compartments which is measured by an intracellular electrode.
Observations regarding the influence of divalent cations on the behaviour of excitable tissue preceded Hodgkin and Huxley's description of the Na\textsuperscript{+} and K\textsuperscript{+} channel. Adrian and Gelfan (1933) noted that lowering external Ca\textsuperscript{2+} caused hyperexcitability in muscle and Brink (1954) suggested, from his studies in nerves, that this increased activity resulted from a decrease in membrane resistance upon exposure to low external Ca\textsuperscript{2+}. High concentrations of external Ca\textsuperscript{2+}, on the other hand, were observed to increase membrane resistance and to stabilize excitable tissues (Weidmann, 1955).

Shortly after Hodgkin and Huxley (1952) described the changes in Na\textsuperscript{+} and K\textsuperscript{+} permeability in squid axon during an action potential Frankenhaeuser and Hodgkin (1957) examined the effects of raising and lowering the concentration of extracellular Ca\textsuperscript{2+} on these permeability changes. They observed that a five-fold reduction of the extracellular Ca\textsuperscript{2+} concentration shifted the conductance-voltage relations for both Na\textsuperscript{+} and K\textsuperscript{+} along the voltage axis in a hyperpolarizing direction and was equivalent to depolarizing the membrane by 10 to 15 mV (Frankenhaeuser & Hodgkin, 1957). No measurable differences were observed in the potential between the bulk intracellular and extracellular solutions. To explain these results Huxley proposed that Ca\textsuperscript{2+} might adsorb to negative charges on the outer surface of the cell thereby altering the electric field within the membrane in a manner which effectively hyperpolarized the cell (Frankenhaeuser & Hodgkin, 1957).

Subsequent evidence suggested that fixed negative surface charges exist on the cytoplasmic face of the membrane as well. Chandler et al. (1965) observed that lowering internal ionic strength in giant axons by reducing intracellular K\textsuperscript{+} from 300 mM to 24 mM with a non-electrolyte shifted the activation and inactivation curve for Na\textsuperscript{+} and K\textsuperscript{+} currents along the voltage axis in a depolarizing direction. They attributed this effect to the presence of a fixed layer of negative charges on the inside of the membrane as these shifts did not occur when K\textsuperscript{+} was replaced with an ionic species such as Na\textsuperscript{+} chloride or choline chloride (Chandler et al., 1965).
Membrane Surface Potential Can Be Described Mathematically

The Gouy-Chapman theory of fixed surface charge, developed during the early part of this century, forms the basis for theoretical discussions of the effect of charge screening on the function of voltage-dependent ion channels. The major assumptions of the Gouy-Chapman theory are: 1) the membrane surface potential results from a uniformly smeared density of fixed charge per unit area; 2) the dielectric constant in the aqueous phase is assumed to be a constant and equal to its bulk value; and, 3) the ions are assumed to be point charges, therefore, only the charge an ion carries and not the ionic species is important in an ion’s ability to effectively screen membrane surface charges (Gilbert & Ehrenstein, 1969; McLaughlin et al., 1971; Hille, 1984). The Gouy-Chapman theory is useful for quantitating in mathematical terms the influence of surface charge on the behaviour of voltage-dependent ion channels. However, the Gouy-Chapman-Stern theory, a modification of the Gouy-Chapman theory, is more biologically relevant because it addresses not only specific binding of cations to fixed negative surface charges but also the establishment of a diffuse double layer of ions in the transition area between the bulk solution and the surface of the membrane through non-specific interactions between cations and negative surface charges on the membrane (Hille, 1968).

Both the Gouy-Chapman theory and the Gouy-Chapman-Stern theory have been used to determine the type and density of charges surrounding voltage sensitive ion channels and to determine the effect of varying the ionic composition of solutions on the membrane surface potential (Gilbert & Ehrenstein, 1969; Hahin & Campbell, 1983; Hille, 1968; Hille et al., 1975; McLaughlin et al., 1971; Mozhayeva & Naumov, 1972 a,b&c).

Different Species Of Divalent Cations Are Not Equally Effective In Shifting The Voltage-Sensitive Parameters of Voltage-Gated Ion Channels

Evidence from several studies suggests that the ability of divalent cations to shift the voltage sensitive parameters both of Na⁺ and K⁺ currents depends on the ionic species
(Århem, 1980; Blaustein & Goldman, 1968; Cukierman & Krueger, 1990; Hille et al., 1975; Mozhayeva & Naumov, 1972c). In most instances transition metal ions are much more effective than alkaline earth metal ions in shifting the voltage sensitive gating parameters of these currents along the voltage axis. This suggests there is some specificity in the interaction between these divalent cations and the ion channels whose behaviour they influence.

$\text{Ca}^{2+}$ and the transition metal ions appear to modulate the behaviour of voltage-activated $\text{K}^+$ currents by binding to sites either near or on the channel protein (Armstrong and Cota, 1991; Begenisich, 1988; Begenisich and Lynch, 1974; Blaustein and Goldman, 1968; Cukierman and Krueger, 1990; Gilly and Armstrong, 1972, Hille et al., 1975). Binding of these cations can in theory alter both channel conductance and gating kinetics.

$\text{Zn}^{2+}$ and $\text{Cd}^{2+}$ slow the kinetics of the $\text{K}^+$ current, reduce the amplitude of the $\text{Na}^+$ current and shift the conductance-voltage relation when applied internally in squid giant axon (Begenisich and Lynch, 1974) or externally to myelinated nerve from *Xenopus laevis* (Århem, 1980). Gilly and Armstrong (1982 a&b) observed that changing the external concentration of $\text{Zn}^{2+}$ slowed the opening kinetics of both the $\text{Na}^+$ and $\text{K}^+$ channels without altering the closing kinetics. Rather than simply interacting with fixed negative surface charges on the external membrane, Gilly and Armstrong (1982 a&b) suggested that $\text{Zn}^{2+}$ interacts directly with a negatively charged element of the gating apparatus and prevents the negative charge from moving inward when the membrane depolarizes thereby stabilizing the channel in the closed position.

Begenisich and Lynch (1974) observed similar effects on the kinetics of $\text{Na}^+$ and $\text{K}^+$ channels of squid giant axon when they increased the internal concentration of $\text{Zn}^{2+}$, $\text{Co}^{2+}$, $\text{Cd}^{2+}$ or $\text{Ni}^{2+}$. Internal $\text{Ca}^{2+}$ concentrations of up to 10 mM had no effect on either current (Begenisich and Lynch, 1974). The latter study also concluded that transition metal ions must interact with membrane constituents involved in the control of gating and found that the $\text{K}^+$ current was more susceptible than the $\text{Na}^+$ current.
Some Species Of Divalent Cations Block Ion Channels In A Voltage-Dependent Manner

In some cases divalent cations reduce the conductance of ion channels by causing a voltage-dependent block of the current. Divalent cations from the alkaline earth metal and the transition metal series were found to cause a voltage-dependent block of TTX-sensitive and -insensitive Na⁺ channels (Schild et al., 1991; Ravindran et al., 1991). Woodhull (1973) observed that Na⁺ channels in frog myelinated axon were blocked by protons and Ca²⁺ in a voltage-dependent manner.

Based on the decrease in Na⁺ conductance under conditions of low external pH, Woodhull (1973) and subsequent investigators have proposed that the proton binding site is a titratable acid moiety, most likely a carboxylic acid, lying far enough across the electric field of the membrane to be affected by the potential difference across the membrane (Campbell, 1982; Campbell & Hille, 1976; Mozhayeva & Naumov, 1981 & 1983; Sigworth, 1980; Yatani et al., 1984). Others have suggested that the binding site responsible for the decrease in Na⁺ channel conductance lies near the outer surface of the channel and that protonation of this site reduces Na⁺ channel conductance due to electrostatic interactions which reduce the concentration of Na⁺ near the entrance of the channel (Drouin & Neumcke, 1974).

Voltage-dependent block by divalent cations or protons does not preclude contributions arising from surface charge interactions. Ravindran et al. (1991) found their results were best fitted when a single divalent binding site was combined with the Gouy-Chapman theory of surface charge. Distinguishing between a voltage-dependent block and screening of fixed surface charges is difficult at the macroscopic current level and both mechanisms may be involved.
Molecular Characterization Of Voltage-Gated Ion Channels

Characterization, at the molecular level, of the proteins which form voltage-gated ion channels has identified specific segments and in some cases individual amino acids within the proteins which play critical roles in determining the gating, conductance, ionic selectivity and pharmacological profile of these channels (reviewed in Betz, 1990 and Jan & Jan, 1989).

Molecular sequencing and subsequent studies utilizing site directed mutagenesis have identified the fourth putative transmembrane segment (the S4 region), which is ubiquitous among all voltage-gated ion channel proteins sequenced, as the most likely candidate for the voltage sensor (Betz, 1990; Catterall, 1988; Jan and Jan, 1989; Kamb et al., 1987; Papazian et al., 1987 & 1988; Stühmer, 1991). The S4 region has a positively charged lysine or arginine at every third amino acid position interspersed between mostly hydrophobic amino acids (reviewed in Betz, 1990; Jan & Jan, 1989 & 1990; and, Unwin, 1989). Based on the presence of the positively charged amino acids in the S4 region several groups have pointed to the involvement of this region in the transfer of charge across the membrane during the voltage-dependent transitions which occur prior to channel activation (Benndorf, 1989; Catterall, 1988; Jan & Jan, 1989). Common to each of these models is the idea that during or prior to channel opening conformational changes of the S4 region result in the net transfer of positive charges across the electric field of the membrane from the intracellular to the extracellular side.

As discussed previously, electrophysiological studies suggest that changes in divalent cation concentration can alter the electric field sensed by this S4 region and thus shift the voltage-dependence of activation. Zn$^{2+}$ and other transition metal ions also appear to interfere specifically with the movement of the S4 region and thus the transfer of charge
across the electric field of the membrane (Gilly and Armstrong, 1982 a&b; Begenisich & Lynch, 1974).

**Is The Behaviour Of The Transient Outward Potassium Channel Influenced By Divalent Cations?**

Most of the work regarding the effects of changing divalent cation concentration on the behaviour of voltage-dependent channels has focused on either Na\(^+\) channels or the delayed rectifier type K\(^+\) channel. Few investigators have addressed how changing divalent cation concentration will affect the behaviour of the transient outward potassium current (TOC) despite the fact that the transient outward potassium channel is the best characterized K\(^+\) channel at the molecular level.

Connor and Stevens (1971) first identified the TOC, which they termed the A-current (I\(_A\)), in the cell body of a gastropod neurone. Pharmacologically, the TOC is often characterized by its sensitivity to block by 4-aminopyridine (Rudy, 1988). The gating behaviour of the TOC resembles that of the voltage-dependent Na\(^+\) current. That is to say, the TOC activates quickly, (0.5-20 ms) and inactivates with a time constant averaging 50 ms (Rudy, 1988). The threshold for activation is around -60 mV for most cells and steady-state inactivation is usually complete at about -40 mV (Rudy, 1988). The TOC often activates only when a cell is depolarized after a period of hyperpolarization (Hille, 1984). The proposed role for the TOC is to prolong the interval between action potentials by opposing the depolarizing effect of inward pacemaker currents (Hille, 1984; Rudy, 1988).

**Melanotrophs Possess A Transient Outward Potassium Current**

Melanotrophs comprise the majority of cells found in the pars intermedia of the pituitary and form part of the pro-opiomelanotropinergic endocrine system (reviewed in O'Donohue & Dorsa, 1982). In the rat, the pars intermedia is well defined and lies in the cleft between the posterior and anterior pituitary. Pro-opiomelanocortin (POMC) is the
common pre-cursor for all peptides synthesized and released by this system (O'Donohue & Dorsa, 1982).

Melanotrophs secrete several peptide hormones, however, α-melanocyte stimulating hormone (α-MSH), which they stain for (Bäck & Rechardt, 1985) is the best known product. α-MSH stimulates melanin production and dispersion in melanocytes and also has several extrapigmentary actions (O'Donohue & Dorsa, 1982). β-adrenergic agonists stimulate and dopamine inhibits the release of α-MSH from melanotrophs (Douglas & Taraskevich, 1978; O'Donohue & Dorsa, 1982). Several other neural inputs are also postulated to regulate secretions from the pars intermedia (de Rijk et al., 1990; Kehl et al., 1987; O'Donohue & Dorsa, 1982).

Melanotrophs generate spontaneous action potentials which are predominantly Na+-dependent but which have a Ca²⁺ component (Douglas & Taraskevich, 1982; Tomiko et al., 1981) and they possess voltage-gated Ca currents (Cota, 1986; Kehl, 1987) which appear to be involved in stimulus-secretion coupling (Tomiko et al., 1981).

Kehl (1989) characterized two voltage sensitive outward K⁺ currents in cultured melanotrophs from the pars intermedia of adult rats. One is a delayed rectifier type K⁺ current, I_K(s), which activates slowly and inactivates slowly (Kehl, 1989). The other, of interest in the context of the present study, is a TOC, I_K(f), which is sensitive to block by 4-aminopyridine (Kehl, 1990). The activation threshold for I_K(f), determined in high external Ca²⁺ (10 mM), was between -20 and -10 mV with steady-state inactivation complete at -10 mV (Kehl, 1989). The time constant for inactivation was between 20-35 ms and was well fitted by a single exponential (Kehl, 1989).

**Experimental Rationale**

Kehl (1989) observed that Cd²⁺ (5 mM) or Co²⁺ (10 mM) reduced the peak amplitude and increased the 50% rise time of I_K(f). Mayer and Sugiyama (1988) reported similar effects of divalent cations on I_A, a TOC in cultured dorsal root ganglion cells from
the rat. They observed that divalent cations evoked a right-ward shift of the activation and inactivation curves along the voltage axis, which accounted for the reduction in current, and suggested that the divalents exert this effect by binding to the channel protein (Mayer & Sugiyama, 1988).

One of the major goals of the present study was to elaborate on the findings of Kehl (1989) and to examine in greater detail the effect of varying the external concentration of Cd²⁺, Zn²⁺, Ca²⁺ and Mg²⁺ on the behaviour of I_K(f). The aim was to determine if these cations interacted directly with the channel protein as appears to be the case in rat sensory neurones (Mayer & Sugiyama, 1988) or if their effects arose indirectly through non-specific screening of fixed negative surface charges.
1. Preparation Of Acutely Dissociated Melanotrophs

Melanotrophs were obtained from adult male Wistar rats (200-300g). Prior to decapitation the rats were exposed to CO\textsubscript{2} until unconscious. The excised pituitary gland was placed in a 1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium (DMEM) at room temperature and the neurointermediate lobe which consists of the pars intermedia and the pars nervosa was separated from the pars anterior. The neurointermediate lobe was then incubated in a 35\degree C water bath for 25-50 min in 0.5 ml Ca\textsuperscript{2+}, Mg\textsuperscript{2+} -free phosphate-buffered saline (CMF-PBS) containing collagenase (type V; 1 mg/ml = 435 active units (U)/ml) and hyaluronidase (type II; 1 mg/ml = 410 U/ml) and for 10-25 min in 0.5 ml CMF-PBS containing protease (type VIII; 0.5-1 mg/ml = 5-15 U/ml). Following enzymatic treatment the tissue was transferred to 0.5 ml of CMF-PBS and mechanically dispersed by trituration through progressively smaller syringe needles (18, 21, 23 & 26 gauge). The cell suspension was then spun at approximately 50 g for 10 min on a column of CMF-PBS containing 10\% (w/v) bovine serum albumin (BSA; fraction V). The supernatant was removed and the cells were resuspended in culture media comprised of Ham's F-12:DMEM (1:1) and kept in an atmosphere- controlled (95% air : 5% CO\textsubscript{2}), humidified incubator at 37\degree C until use (1-15 hours).

For each experiment an aliquot of the cell suspension was transferred to a Perspex chamber mounted on the stage of an Olympus inverted phase contrast microscope. The cells were viewed at 600X magnification. Melanotrophs were identified as those cells which exhibited phase-bright membranes, contained dark inclusions (assumed to be the nucleus) and were 10-15 \textmu m in diameter. Occasionally there were pleomorphic cells present which were assumed to be pituicyles and small enucleate vesicles which were assumed to be either detached nerve terminals from the pars nervosa or vesicles formed spontaneously from the lipid of ruptured membranes.
2. Electrophysiology

Macroscopic ionic currents were recorded via conventional patch-clamp techniques utilizing the whole-cell voltage clamp configuration (Hamill et al., 1981). A LIST EPC-7 patch-clamp amplifier was used to measure the whole-cell currents which were then encoded (Instrutech VR-10, Elmont, NY) and stored on video cassette tape (3 dB at 10 kHz). The voltage commands were derived from a custom-built digital potentiometer controlled by a custom-built digital timer. All experiments were conducted at room temperature (20-25°C). Control, test and recovery responses were determined for each cell examined so that each cell functioned as its own control.

Patch electrodes made of borosilicate glass (Corning No. 7052, A-M Systems, WA, USA) were pulled on a Narishige PP-83 two stage puller. The electrode tip outer diameter was 1.5-2 µm prior to fire polishing. The signals were referenced to an agar salt bridge (4% agar by weight in 150 mM NaCl) and the zero-current voltage was set on the EPC-7 once the electrode tip was immersed in the bath solution. The resistance of the fire-polished electrode tip, measured in the external control solutions, varied from 3-7 MΩ. The tip was coated with Sylgard (Dow Corning) to reduce the capacitive transients.

The formation of a tight (gigohm) seal and the establishment of the whole-cell configuration was usually accomplished in the external control solutions described below. In some cases a modified external solution containing 10 mM Ca²⁺ was used to obtain the whole-cell configuration as high Ca²⁺ appeared to promote tight seal formation. As soon as the whole-cell recording was established the external solution was changed to the external control solution.

Capacitive transients arising from the cell and the pipette were compensated using circuitry incorporated in the EPC-7. The series resistance ($R_s$) for the whole-cell recording was typically 16-25 MΩ and was compensated only if the macroscopic currents were large. $R_s$ compensation was usually 30-60%, which, with a 1 nA current (upper limit of those
examined), would result in at most a 17 mV error in the voltage command. There was no compensation for any such error.

The stimulus frequency was 0.25 Hz and the holding potential ($V_H$) was -80 mV unless otherwise noted.

3. Data Acquisition and Analysis

For analysis, current signals were passed through a 4-pole low-pass Bessel filter with a -3 dB cut-off frequency of 2 kHz and digitized at a sampling frequency at least twice the filter cut-off frequency using a 12-bit analog/digital converter (Scientific Solutions Labmaster, OH) interfaced with an AT clone. Current and potential recordings were analyzed off-line with an Intel-based computer using BASIC-Fastlab routines (INDEC Systems, Sunnyvale, Calif.).

$I_K(f)$ was separated from the leak and residual $I_K(s)$ currents using a two-pulse protocol. When a two pulse protocol was used the first pulse is referred to as the conditioning pulse and the second pulse in referred to either as the conditioned or non-conditioned test pulse. No less than three current traces were averaged for each conditioned and non-conditioned test pulse. A non-conditioned 350 ms test pulse from the holding potential to -40 mV or more elicited an outward current composed of $I_K(f)$, residual $I_K(s)$ and leak current. Since the time dependence of inactivation determined at -40 mV was well fitted by a single exponential with a time constant ($\tau$) of 54.7 ms a conditioning pulse length of 600 ms (approximately 11 time constants) was judged sufficient to virtually eliminate $I_K(f)$. Consequently it was possible to isolate $I_K(f)$ by subtracting the averaged conditioned pulse from the averaged non-conditioned pulse. For measuring purposes $I_K(f)$ was defined as the difference between the peak outward current and the steady-state current.

Curve fitting was done by using non-linear regression routines in BASIC-Fastlab. Values are expressed as the mean ± the standard error of the mean (s.e.m.) and "n"
represents the number of cells studied. Statistical analysis of the results was completed using SAS (Cary, NC). Where there were more than two concentrations of a given divalent cation being examined, analysis of variance (ANOVA) was employed with \( p = 0.01 \). If only one concentration of a given divalent cation was tested, significance was determined by using a paired t-test, again with \( p = 0.01 \).

4. Recording Solutions

The compositions of the external solutions are summarized in Table 1. The pH of each of these solutions was adjusted to 7.4 with 1 M NaOH. Tetraethylammonium chloride (TEA) was included to block the slowly-activating, slowly-inactivating \( K^+ \) current (\( I_{K(s)} \); Kehl, 1989), and tetrodotoxin (TTX; 1-2 \( \mu \)M) was included to block inward Na\(^+ \) currents (McBurney & Kehl, 1988). \( \text{Cd}^{2+} \) was not included in the standard external solution to block Ca\(^{2+} \) currents because even at the low concentrations (300 \( \mu \)M) necessary, \( \text{Cd}^{2+} \) affected the kinetics and conductance-voltage relation of \( I_{K(f)} \). Inward Ca\(^{2+} \) currents are normally much smaller than the outward \( K^+ \) currents and in almost all cells examined inward Ca currents were not detected.

In some cases sucrose was added to the external solutions to make them hyperosmotic relative to the pipette solutions as this appeared to aid in tight seal formation and in prolonging the membrane integrity of the cell once whole-cell recording was established.

For some of the experiments a three-buffer system was utilized in which 10 mM HEPES was replaced by 5 mM HEPES, 5 mM CHES (2-[N-cyclohexylamino] ethanesulfonic acid) and 5 mM propionic acid-Na salt. In these cases a three-buffer system was also employed in the pipette-filling solution (see below). The three-buffer solutions were prepared in order to conduct pH experiments which are not discussed here. \( I_{K(f)} \) recorded in the three-buffered solutions at pH 7.4 exhibited the same behaviour as in HEPES
buffered solutions at pH 7.4. For subsequent divalent cation experiments the more conventional HEPES buffered solutions were prepared.

The standard patch pipette solution contained (in mM): 140 KCl, 5 MgCl₂-6H₂O, 1 CaCl₂, 10 HEPES, 11 EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 1 Na₂ATP; pH was adjusted to 7.4 with 1M KOH, pCa>8. For the experiments in which a three-buffer system was used, 10 mM HEPES in the pipette solution was replaced by 5 mM HEPES, 5 mM CHES and 5 mM propionic acid-Na⁺ salt. The standard HEPES buffered pipette solution is designated "1" and the three-buffer pipette solution is designated "3" in the figure legends. Pipette solutions were passed through a 0.2 μm filter prior to filling the electrode. The term "pipette solution" is used interchangeably with "internal solution" because in whole-cell recording the pipette solution diffuses into the cell to become the internal solution.

The following convention will be used to describe the solutions used for each experiment in the figure legends: external///internal. External solutions are referred to by the letter assigned to them in Table 1 and the concentration (in mM or μM) of the divalent cation of interest will be given. The pipette solutions will be referred to by their number code as described above. If the external solution was changed during the experiment the following convention will be used in the figure legends: control//test [divalent cation]///internal.

The Na salt of Phenol Red (200 μM; Sigma, tissue culture grade) was added to the test solutions so that solution exchanges in the recording chamber could be monitored visually. Solution exchange was accomplished using a stop-flow system in which the test solution flowed into one end of the recording chamber while solution was drawn off at the other end of the chamber at a rate sufficient to maintain a constant fluid level in the bath. Solution exchange continued until the solution in the bath was completely replaced and any changes in the behaviour of the current, evoked as a result of changes in the solution
composition, had reached a steady-state - usually within 1-2 min - after which the flow was stopped and the recording continued.

Chemicals were obtained either from Sigma or Aldrich. Solutions were stored at 4°C and allowed to warm to room temperature prior to use.
<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>126</td>
<td>112.5</td>
<td>112.5</td>
<td>120</td>
<td>120</td>
<td>126</td>
<td>126</td>
<td>120</td>
<td>123</td>
<td>63</td>
</tr>
<tr>
<td>KCl</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>TEA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>TTX (μM)</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>CHES</td>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic Acid-Na</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>5-</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>CdCl₂</td>
<td></td>
<td>0.1</td>
<td></td>
<td>2</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnCl₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.0031</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.1%</td>
<td>.1%</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
RESULTS

SECTION I: GENERAL PROPERTIES OF $I_K(f)$

1. Voltage- and Time-Dependence of Activation

The activation threshold for $I_K(f)$ under control conditions was between -40 and -30 mV and the peak amplitude of the current increased monotonically with increasing membrane depolarization (Fig. 1a,b) as previously reported (Kehl, 1990). From -30 mV to +60 mV the current-voltage relation was non-linear but approached linearity at membrane potentials above +60 mV as the maximum conductance ($G_{max}$) was reached (Fig. 1b).

The data points (filled circles) in Fig. 1d indicate the normalized chord conductance ($g$) derived from the $I_K(f)$ at each test potential. The reversal potential of -65 mV determined in standard control solution (discussed in detail in Section I part 4) was used to calculate the chord conductance. The curve fitted to the data points by a least squares regression routine to the Boltzman equation,

$$g/G_{max} = [1 + \exp((V' - V)/k)]^{-1},$$

indicated that half-activation ($V'$) occurred at -13.6 mV with a slope-factor ($k$) of +12.8 mV ($n=55$).

The slope of the conductance-voltage relation, which represents the voltage-sensitivity of activation gating, can be converted to an equivalent gating charge by solving for $Z$, the effective valency of the gating particle, in the relationship,

$$k = KT/Ze,$$

where $k$ is the slope-factor, $K$ is Boltzman’s constant ($1.38 \times 10^{-23} \text{ J/K}$), $T$ is the absolute temperature and $e$ is the size of the electronic charge ($1.6 \times 10^{-19} \text{ C}$). At 20°C, $KT/e$ is approximately 25 mV. Solving for $Z$ indicates that an equivalent of 2 gating charges must move completely across the membrane field during activation.
The rate of rise of $I_K(f)$ was strongly influenced by depolarization (Fig. 1a). Under control conditions, the 50% rise time ($t_{\text{rise}}$) decreased from $2.8 \pm 0.1 \text{ ms} \ (n=20)$ at -20 mV to $0.8 \pm 0.1 \text{ ms} \ (n=30)$ at +60 mV in agreement with previous results (Kehl, 1989).

2. Voltage- and Time-Dependence of Inactivation

Steady-state inactivation was analyzed by using a two-pulse protocol in which the cell was held at -80 mV and stepped to 0 mV for 350 ms immediately after a 600 ms conditioning pulse of varying intensity. The conditioned test currents for one cell are shown in Fig. 1c. In Fig. 1d the peak current measured during the conditioned test pulse is plotted against the pre-pulse potential. The curve fitted to the data points (open circles) by a least squares regression routine represents the solution for the Boltzmann equation,

$$\frac{I}{I_{\text{max}}} = [1 + \exp((V-V')/k)]^{-1},$$

and indicates that half-inactivation ($V'$) occurred at -54.7 mV. As discussed previously, the slope-factor ($k$) of -4.4 mV ($n=50$) reflects the voltage sensitivity of inactivation gating. Determination of the equivalent gating charge for inactivation indicates that the equivalent of 6 gating charges moves across the membrane electric field during inactivation suggesting that inactivation is more steeply voltage-dependent than activation.

At -20 mV, the decay of $I_K(f)$ was well fitted by least squares regression analysis to a single exponential with a time constant of decay ($\tau$) of $25.8 \pm 1.1 \text{ ms} \ (n=10)$ as shown for one cell in Fig. 2a. At potentials above -20 mV, the decay of $I_K(f)$ was often better fitted by two exponentials as illustrated for one cell in Fig. 2b. The slow component at +60 mV was $66.2 \pm 1.8 \text{ ms} \ (n=10)$ and accounted for $66 \pm 1.6\%$ of the decay, whereas the fast component at +60 mV was $14.2 \pm 2.3 \text{ ms} \ (n=10)$ and accounted for $34 \pm 1.6\%$ of the decay.
Figure 1. The current-voltage relation and steady-state inactivation of $I_{K}(f)$ under control conditions. A. Whole-cell currents recorded during 350 ms steps to -20, 0, +20, +40 and +60 mV from a holding potential of -80 mV. B. The current-voltage relationship for $I_{K}(f)$. Data points represent the peak amplitude of $I_{K}(f)$, as defined in the methods, at each potential. C. Superimposed currents during test pulses to 0 mV following 600 ms conditioning pulses to -100, -80, -60, -55, -50, -45 and -30 mV; the current traces following pulses to -100 and -80 mV are superimposed. The current traces were not leak subtracted. D. The control activation (filled circles) and steady-state inactivation (open circles) curves for $I_{K}(f)$. For the activation curve the normalized conductance was plotted against the membrane potential during the test pulse. The curve represents the solution to the Boltzman equation where the potential for half-activation was -15.5 mV and the slope-factor was 10.3 mV. The inactivation curve is the normalized test current plotted against the membrane potential during the conditioning pulse. The half-inactivation potential was -55.6 mV and the slope-factor was -3.7 mV. The straight line in A and C represents the zero current level. Cell 160492B. Recording solutions $E///1$ (see Table 1).
3. **Voltage-Dependence of the Residual Steady-State Current**

   The magnitude of the time-dependent residual steady-state current (e.g., Fig. 1d) increased with membrane depolarization. This current probably arises from \( I_{K(s)} \) channels which are not blocked in 20 mM TEA. Some evidence suggests that the block of delayed rectifier type \( K^+ \) channels by TEA is voltage-dependent (Hille, 1984; Clay, 1985) and this might account for the observation that the residual steady-state current increases with depolarization.

4. **Reversal Potential For \( I_{K(f)} \)**

   The reversal potential for \( I_{K(f)} \) was determined by using a two pulse protocol. A conditioned pulse to 0 mV for 10 ms maximally activated \( I_{K(f)} \) but produced very little inactivation of the current. This pulse was immediately followed by a test pulse to the potentials indicated beside the current traces in Fig. 3. At maximal activation a majority of the channels are open and the test pulse will drive current through the open channel in a direction which is dictated by the difference between the membrane potential and the equilibrium potential for the current. The potential at which no time-dependent current is evident is taken to be the reversal potential (\( E_R \)). This was measured either directly or by interpolation. It should be noted that a time-independent current (the leak current) also contributes to the tail current. \( E_R \) varied from -60 to -70 mV among the cells examined. For the purposes of calculating the chord conductance an \( E_R \) of -65 mV was chosen as this value fell between the extremes. \( E_K \), calculated from the Nernst Equation to be -93.2 mV, was substantially more hyperpolarized than \( E_R \). This suggests that the channel conducting \( I_{K(f)} \) is not completely selective for \( K^+ \).
Figure 2. The time constants for the inactivation of $I_{K}(f)$. A. The decay of the current evoked at -20 mV was well fitted by a single exponential with $\tau = 25.07$ ms. B. The decay of the current at +60 mV was better fitted by two exponentials. The slow component ($\tau_{\text{slow}}$) was 61.4 ms and accounted for 63% of the current decay and the fast component ($\tau_{\text{fast}}$) was 14.1 ms and accounted for 37% of the current decay. The curve representing the sum of the fast and slow components of decay overlays the current trace. Separate curves representing the fast and slow components are illustrated by the dashed lines. Cell 140492A. Recording solutions E///1.
Figure 3. The reversal potential for $I_K(f)$ as determined from tail currents following a 10 ms pulse to 0 mV. The potential at which each current was recorded is indicated beside the trace. The trace which exhibited no time-dependent current illustrates the $E_R$. The estimated $E_R$ is indicated by an arrow. $V_h$ was -80 mV. Cell 140591A. Solutions: B//3.
SECTION II: THE EFFECTS OF DIVALENT CATIONS ON I\(_K(f)\)

Divalent cations have been shown to shift the activation and inactivation curves of voltage sensitive Na\(^+\) and K\(^+\) channels. This effect of divalent cations is attributed to either the non-specific screening of fixed negative surface charges or the specific interaction of these cations with sites close to or on the channel protein. The purpose of the present study was to elaborate on the observations of Kehl (1989) and to determine whether divalent cations affect I\(_K(f)\) in a manner analogous to that reported for I\(_A\), a TOC in rat sensory neurones (Mayer & Sugiyama, 1988).

1. THE EFFECTS OF TRANSITION METAL IONS ON THE GATING OF I\(_K(f)\)

1.1 Cadmium

Using a single concentration of Cd\(^{2+}\) (5 mM) Kehl (1989) observed that there was a reduction of the peak amplitude of I\(_K(f)\) and an increase of the 50% rise time of the current. This effect was examined further over a range of Cd\(^{2+}\) concentrations.

1.11 Cd\(^{2+}\) Reduces The Peak Amplitude Of I\(_K(f)\)

The concentration-dependent reduction of the peak amplitude of I\(_K(f)\) by Cd\(^{2+}\) (100 \(\mu\)M to 1600 \(\mu\)M), is illustrated in the current-voltage relations summarized in Fig. 4a where the data points represent the means from 5 cells. The reduction of peak I\(_K(f)\) by Cd\(^{2+}\) was particularly noticeable at 0 mV and traces illustrating the dose-dependent decline of I\(_K(f)\) at this potential are presented in Fig. 4b. Overall, the peak amplitude of I\(_K(f)\) at 0 mV was reduced 25 ± 2.6%, 37 ± 2.2%, 50 ± 2.4%, 63 ± 2.0% and 73 ± 1.5% by a Cd\(^{2+}\) concentration of 100, 200, 400, 800 and 1600 \(\mu\)M, respectively, (n=5; e.g., Fig. 4b).

The effect of Cd\(^{2+}\) on the peak amplitude of I\(_K(f)\) was less pronounced at more depolarized potentials. The relationship between the reduction of I\(_K(f)\) at +60 mV and the external concentration of Cd\(^{2+}\) is illustrated in Fig. 4c. The line fitted to the data points represents the best fit to the Michaelis-Menton equation, \(R = R_{\text{max}}/(1+(K_M/[\text{Cd}^{2+}])^n)\),
Figure 4. The concentration-dependent effects of Cd$^{2+}$. A. Normalized current-voltage relations for $I_K(f)$ in control (open circles) and in the presence of 100 µM (crosses), 200 µM (diamonds), 400 µM (squares), 800 µM (triangles) and 1600 µM Cd$^{2+}$ (filled circles). Data points represent the mean for five cells. $I_K(f)$ was normalized against the maximum current obtained in control. B. Superimposed whole-cell currents evoked by a 350 ms test pulse to 0 mV from the holding potential illustrating the dose-dependent reduction of $I_K(f)$ by Cd$^{2+}$. Also apparent from these traces is the slowing of the activation rate as the concentration of Cd$^{2+}$ is increased. The concentration of Cd$^{2+}$ is indicated beside the individual traces. The straight line below the current traces identifies the zero current level. C. A concentration-response curve comparing the reduction of normalized $I_K(f)$ ($1-I_{\text{norm}}$) to Cd$^{2+}$ concentration. Data points represent the mean ± s.e.m. for five cells. The curve fitted to the data points is a solution to the Michaelis-Menton equation assuming a single binding site where the maximum current reduction is 38.4% and the $K_M$ for the effect of Cd$^{2+}$ is 256 µM. D. The dose-dependent shift of the activation curve by Cd$^{2+}$ in which normalized conductance is plotted against the test potential for each concentration of Cd$^{2+}$ (symbols represent the same Cd$^{2+}$ concentrations as for A above). The chord conductance was determined at each potential in each concentration of Cd$^{2+}$. Curves were normalized to the maximum conductance at each concentration of Cd$^{2+}$ to correct for possible channel block (Perozo & Bezanilla, 1990). Curves fitted to the data points represent solutions to the Boltzman equation where the potentials for half-activation, in ascending order from control to 1600 µM Cd$^{2+}$, were -11.9 mV, -5.0 mV, -0.9 mV, 4.1 mV, 10.1 mV and 12.3 mV, respectively. The slope-factor for these activation curves did not appear to be altered by the presence of Cd$^{2+}$ and was approximately 12.4 mV. Cell 040991C. Recording solutions B//C: [Cd$^{2+}$] indicated below///3.
where n is assumed to be 1. From the fitted curve, the reduction (R) of the normalized current at +60 mV saturated (R_max) at 38.4% and the concentration of Cd²⁺ at which half-maximal reduction occurred (K_M) was 255.8 μM (n=5). For the five cells examined, the reduction of I_K(f) by Cd²⁺ at +60 mV ranged from 13 ± 2.3% in 100 μM Cd²⁺ to 35 ± 2.3% in 1600 μM Cd²⁺ (Fig. 4c).

Following the analysis of the dose-response to Cd²⁺, the effects of 500 μM, 2 mM and 5 mM Cd²⁺ on I_K(f) were more thoroughly examined. At a concentration of 500 μM, Cd²⁺ caused a rightward shift of the current-voltage relation for I_K(f) along the voltage axis. The reduction of peak I_K(f) and the shift of the threshold for activation from -40 mV to -20 mV caused by 500 μM Cd²⁺ are illustrated for a representative cell in Fig. 5a. Overall, 500 μM Cd²⁺ reduced the peak I_K(f) by 50 ± 3.8% at 0 mV and 9 ± 2.2% at +60 mV (n=5) and caused a slight, 4 ± 2.7% (n=5), reduction of G_max. For the cell shown in Fig. 5a G_max was reduced from 13.6 nS to 12.3 nS. The reduction of peak I_K(f) and G_max in the presence of 500 μM Cd²⁺ is most likely due to the right-ward shift of the activation curve. The effects of 500 μM Cd²⁺ were completely reversed within two minutes of returning to Cd²⁺-free control solution (e.g., filled circles; Fig. 5a).

As expected from the apparent saturation of the Cd²⁺ effect at 1.6 mM in the dose-response experiments (see Fig. 4c), there were no measurable differences in the effects exerted by 2 mM and 5 mM Cd²⁺ on I_K(f). For this reason, only the results for 5 mM Cd²⁺ are discussed in detail here. Fig. 5b illustrates the effect of 5 mM Cd²⁺ on a representative cell. At a concentration of 5 mM, Cd²⁺ shifted the threshold for activation of I_K(f) from -40 mV to 0 mV. In the 8 cells tested the peak amplitude of the current was decreased 90 ± 2.0% at 0 mV and 30 ± 4.2% at +60 mV and G_max was reduced by 23 ± 4.4% (n=8) in the presence of 5 mM Cd²⁺. For the cell illustrated in Fig. 5b, G_max decreased from 9.8 nS to 7.2 nS.
Figure 5. The effects of 500 μM and 5 mM Cd²⁺ on the voltage-dependence of activation and inactivation for Iₖ(f). A. Effect of 500 μM Cd²⁺ on the normalized current-voltage relation for Iₖ(f) in control (open circles), 500 μM Cd²⁺ (diamonds) and recovery (filled circles). Cell 140492C. Solutions: E//E&F combined to [Cd²⁺]=500 μM//1. B. As for A but with 5 mM Cd²⁺. Solutions: E//F//1. C. The activation and steady-state inactivation curves for Iₖ(f) in control (filled and open circles, respectively) and in the presence of 500 μM Cd²⁺ (filled and open diamonds, respectively). Same cell as in A. V’ was -11.5 mV in control and shifted to +9.6 mV in the presence of 500 μM Cd²⁺. The slope-factor increased from a control value of 11.5 mV to 12.2 mV. From the inactivation curves, V’ was -46.5 mV in control and shifted to -27.6 mV in control. In the presence of 500 μM Cd²⁺ there was little change in the slope-factor which was -3.9 mV in control and -3.4 mV in 500 μM Cd²⁺. D. As for C but with 5 mM Cd²⁺. Same cell as in B. The half-activation potential, was -10.9 mV in control and shifted to +24.5 mV in 5 mM Cd²⁺. The slope-factor remained relatively constant, with a value of 11.4 mV in control and 11.2 mV in the test conditions. For steady-state inactivation, the half-inactivation potential shifted from -50.1 mV in control to -15.3 mV in 5 mM Cd²⁺. The slope-factor was -3.4 mV under both conditions.
1.12 Cd\(^2+\) Causes A Right-Ward Shift Of The Activation Curve

The voltage-dependent reduction of I\(_K(f)\) observed in the presence of Cd\(^2+\) is explained in part by the Cd\(^2+\)-induced right-ward shift of the activation curve along the voltage axis. The values for V' and k, (estimated as described in Section I), were determined in each concentration of Cd\(^2+\) tested and are summarized in Table 2 (pp 42-43). The magnitudes of the shifts of the half-activation potential (V') with respect to Cd\(^2+\) concentration are summarized in Table 6 (pp 79-80).

The progressive right-ward shift of the conductance-voltage relation as the concentration of Cd\(^2+\) was increased from 100 \(\mu\)M to 1600 \(\mu\)M is illustrated for one cell in Fig. 4d. In the concentration-response experiments (n=4), V' ranged from -12.9 ± 0.6 mV in control (e.g., circles, Fig. 4d) to -4.4 ± 0.5 mV in 100 \(\mu\)M Cd\(^2+\) (e.g., crosses, Fig. 4d) and +11.4 ± 1.3 mV in 1600 \(\mu\)M Cd\(^2+\) (e.g., filled circles, Fig. 4d; values for other concentrations are summarized in Table 2). The slope-factor of the conductance-voltage relation increased from the control value of 15.7 ± 0.3 mV to 11.4 ± 0.2 mV in 100 \(\mu\)M Cd\(^2+\), the lowest concentration of Cd\(^2+\) tested; however, although there was some variability in k values at other Cd\(^2+\) concentrations, increases of the Cd\(^2+\) concentration beyond 100 \(\mu\)M did not further increase the slope of the conductance-voltage relation (see Table 2).

In 500 \(\mu\)M Cd\(^2+\), V' for the conductance-voltage relation shifted +24.6 ± 2.3 mV (p<0.01), from -13.7 ± 1.6 mV in control responses (filled circles) to 10.9 ± 1.7 mV (n=5; filled squares) (Fig. 5c). The slope-factor (k) did not change in the presence of 500 \(\mu\)M Cd\(^2+\) (see Table 2).

At a concentration of 5 mM, Cd\(^2+\) shifted the activation curve +29.2 ± 3.7 mV (n=7; p<0.01; see Table 6), as V' shifted from a control value of -7.4 ± 2.6 mV (filled circles, Fig. 5d) to +21.8 ± 1.1 mV (filled diamonds, Fig. 5d). The slope-factor estimated from the activation curve decreased from 12.3 ± 0.5 to 11.1 ± 0.4 (Table 2) in 5 mM Cd\(^2+\).
1.13 Concentration Dependence For Cd\textsuperscript{2+}-Induced Shifts Of V' 

The relationship between the shift of V' (estimated as described in Section I, Fig. 1) and the external Cd\textsuperscript{2+} concentration is illustrated in Fig. 6. The line fitted to the data points represents the best fit to the Michaelis-Menton equation,

\[ S = \frac{S_{\text{max}}}{1 + (K_M/[Cd^{2+}])^n}, \]

in which n is assumed to be one. From the fitted curve, the shift (S) of V' for the conductance-voltage relation, caused by Cd\textsuperscript{2+}, saturated (S\text{max}) at +28 mV and the concentration of Cd\textsuperscript{2+} at which the half-maximal shift occurred (K_M) was 221 \mu M. This value for K_M is in close agreement with the K_M value (256 \mu M) estimated from the reduction of peak I_K(f) by Cd\textsuperscript{2+}.

1.14 Cd\textsuperscript{2+} Increases The 50% Rise Time (t\text{\%act}) Of I_K(f) 

The time to half-activation was compared since the precise timing of the current peak was ambiguous in the presence of Cd\textsuperscript{2+} (especially at potentials close to the activation threshold) and this made an accurate determination of time to peak I_K(f) difficult. The 50% rise times of I_K(f) in the presence of 500 \mu M (diamonds) and 5 mM Cd\textsuperscript{2+} (squares) and their respective controls (open and filled circles) were compared over a 60-80 mV range and are summarized in Fig. 7a (pp 40-41). As the membrane potential was increased to more depolarized levels there was a decrease in the rise time in control and treated responses. In control conditions, there was approximately a 4-fold decrease of t\text{\%act} at +60 mV (0.7 ± 0.1 ms & 0.6 ± 0.1 ms) compared to t\text{\%act} at -20 mV (2.6 ± 0.1 ms). Cd\textsuperscript{2+} appeared to augment these differences. In 500 \mu M Cd\textsuperscript{2+}, t\text{\%act} decreased approximately 8-fold from 10.1 ± 1.4 ms at -20 mV to 1.2 ± 0.7 ms (n=5) at +60 mV and in 5 mM Cd\textsuperscript{2+}, t\text{\%act} decreased approximately 11-fold from 16.5 ± 2.9 ms at 0 mV to 1.6 ± 0.1 ms (n=5) at +60 mV.
Figure 6. Concentration-dependent effects of Cd$^{2+}$ on the shift of the half-activation potential. Voltage shifts of the half-activation potential as summarized in Table 6 are plotted as a function of the Cd$^{2+}$ concentration. Data points represent the mean ± s.e.m. for the number of cells tested at each concentration of Cd$^{2+}$, "n" is indicated in brackets on the figure. A concentration-response curve fitted to the Michaelis-Menton equation indicates a maximum Cd$^{2+}$-induced shift of +28 mV and a $K_M$ of 220 μM.
The Cd\(^{2+}\)-induced decreases in the activation rate of \(I_{K}(f)\) are readily apparent from the current traces of Fig. 7c,d which show superimposed the control current traces and the current traces for 500 \(\mu\)M Cd\(^{2+}\) and 5 mM Cd\(^{2+}\), respectively. The current trace recorded in 500 \(\mu\)M Cd\(^{2+}\) at 0 mV was scaled up by 2.25 in order to allow a comparison of the rising phase of the current. In 5 mM Cd\(^{2+}\) current traces at +20 mV were compared because the activation threshold for \(I_{K}(f)\) was shifted to 0 mV. The current trace recorded in 5 mM Cd\(^{2+}\) at +20 mV was scaled up by 2.71.

At membrane potentials close to the activation threshold, Cd\(^{2+}\) substantially slowed the 50% rise time of \(I_{K}(f)\). At -20 mV, 500 \(\mu\)M Cd\(^{2+}\) increased \(t_{n\text{act}}\) 3.9-fold, from 2.6 ± 0.1 ms in control to 10.1 ± 1.4 ms (n=5) and at 0 mV, 5 mM Cd\(^{2+}\) increased \(t_{n\text{act}}\) 9.1-fold from 1.8 ± 0.3 ms in control to 16.5 ± 2.9 ms (n=5). At +60 mV, 500 \(\mu\)M Cd\(^{2+}\) increased \(t_{n\text{act}}\) 1.7-fold and 5 mM Cd\(^{2+}\) increased \(t_{n\text{act}}\) 2.6-fold compared to control values (n=5).

1.15 Cd\(^{2+}\) Causes A Right-Ward Shift Of The Steady-State Inactivation (\(h_{o}\)) Curve

The effects of Cd\(^{2+}\) on steady-state inactivation (\(h_{o}\)) were examined at 500 \(\mu\)M, 2 mM and 5 mM Cd\(^{2+}\) and the values for \(V'\) and \(k_{o}\), (estimated as described in Section I) are summarized in Table 2. The magnitudes of the shifts of \(V'\) for the \(h_{o}\) curve with respect to Cd\(^{2+}\) concentration are summarized in Table 6. As previously mentioned, the effects of 2 mM Cd\(^{2+}\) on \(I_{K}(f)\) were the same as those reported for 5 mM Cd\(^{2+}\).

Steady-state inactivation was examined by using a two pulse protocol as discussed in Section I. The data points, for the responses in control (open circles) and 500 \(\mu\)M Cd\(^{2+}\) (open diamonds) are summarized for one cell in Fig. 5c. Overall, curves fitted to the data points (as described in Section I) indicated the \(h_{o}\) curve shifted +21.2 ± 3.9 mV (n=5; \(p<0.01\)) from a \(V'\) of -52.4 ± 1.9 mV in control to -31.3 ± 2.0 mV in 500 \(\mu\)M Cd\(^{2+}\). The slope-factor of the inactivation curve decreased from -4.1 ± 0.2 to -3.4 ± 0.1 (see Table 2) which reflects an increase in the voltage-sensitivity of inactivation.
The data points, for the responses in control (open circles) and 5 mM Cd\(^{2+}\) (open diamonds), comparing the conditioning pulse potential to the peak current elicited during a subsequent test pulse to +30 mV are summarized for one cell in Fig. 5d. The \(h\) curve, fitted as described in Section I, shifted +32.8 mV (n=4; \(p<0.01\)) with \(V'\) increasing from a control value of -48.4 ± 3.6 mV to -15.6 ± 0.3 mV in 5 mM Cd\(^{2+}\). The slope of the inactivation curve did not change with 5 mM Cd\(^{2+}\) present (see Table 2).

1.16 Cd\(^{2+}\) Increases The Time To Half-Inactivation (\(t_{\text{1/2 inact}}\))

The times from the peak of the current to half-inactivation were compared rather than the time constants for decay because at some potentials the decay was well fitted by two exponentials and at other potentials by a single exponential as illustrated in Section I. In Fig. 7d the \(t_{\text{1/2 inact}}\) times are plotted against the membrane voltage for 500 µM Cd\(^{2+}\) (diamonds) and its control (open circles) and from 0 mV to +60 mV for 5 mM Cd\(^{2+}\) (squares) and its control (filled circles).

In general, the Cd\(^{2+}\)-induced increase in \(t_{\text{1/2 inact}}\) was not as great as the increase of \(t_{\text{1/2 act}}\). Moreover, the increase of \(t_{\text{1/2 inact}}\) by Cd\(^{2+}\) did not appear to be concentration-dependent in the range of concentrations used, as shown in Fig. 7b. In the presence of 500 µM Cd\(^{2+}\), \(t_{\text{1/2 inact}}\) at 0 mV increased 1.6-fold from 20.9 ± 0.8 ms in control to 29.2 ± 0.9 ms (n=5) and in 5 mM Cd\(^{2+}\), \(t_{\text{1/2 inact}}\) increased 1.9-fold from 18.6 ± 1.4 ms in control to 36.0 ± 6.3 ms (n=5). At +60 mV, 500 µM Cd\(^{2+}\) increased \(t_{\text{1/2 inact}}\) 1.2-fold from a control value of 30.5 ± 1.4 ms to 36.3 ± 2.6 ms and 5 mM Cd\(^{2+}\) increased \(t_{\text{1/2 inact}}\) 1.3-fold from 29.2 ± 1.0 ms in control to 39.0 ± 2.5 ms (n=5).

1.2 Zinc

Zn\(^{2+}\) has been reported to exert effects on voltage-gated channels in a manner qualitatively similar to that of Cd\(^{2+}\) (Århem, 1980; Begenisich & Lynch, 1975; Gilly &
Armstrong, 1982 a&b). Therefore, the following experiments were undertaken to examine the effect of \( \text{Zn}^{2+} \) on \( I_{K}(f) \).

### 1.21 \( \text{Zn}^{2+} \) Reduces The Peak Amplitude Of \( I_{K}(f) \)

The normalized current-voltage relations from three representative cells in Fig. 8 illustrate the concentration-dependent reduction of \( I_{K}(f) \) by \( \text{Zn}^{2+} \). The reduction of \( I_{K}(f) \) was less pronounced at more depolarized potentials as was the case for \( \text{Cd}^{2+} \). An external \( \text{Zn}^{2+} \) concentration ([\( \text{Zn}^{2+} \)]\( _{o} \)) of 31 \( \mu \)M (e.g., squares, Fig. 8a) reduced \( I_{K}(f) \) by 27 ± 1.8% at 0 mV and by 16 ± 1.5% at +60 mV (n=3). (In one cell examined at 31 \( \mu \)M \( \text{Zn}^{2+} \) there was a 9% decrease at 0 mV and a 7% increase in \( I_{K}(f) \) at +60 mV; this cell was not included in the calculation of the mean). Raising the [\( \text{Zn}^{2+} \)]\( _{o} \) to 62.5 \( \mu \)M (e.g., triangles, Fig. 8a) reduced \( I_{K}(f) \) an additional 7.0 ± 2.0% at 0 mV but had no additional effect at +60 mV. In two cells examined, there was in 62.5 \( \mu \)M \( \text{Zn}^{2+} \), respectively, a 18% and 71% reduction of \( I_{K}(f) \) at 0 mV and a 17% increase and 61% decrease at +60 mV; these cells were not included in the calculation of the mean. Increasing [\( \text{Zn}^{2+} \)]\( _{o} \) to 125 \( \mu \)M (e.g., diamonds, Fig. 8a) reduced \( I_{K}(f) \) by 55 ± 1.2% at 0 mV and by 23 ± 0.5% at +60 mV (n=3) and in one cell examined, 250 \( \mu \)M \( \text{Zn}^{2+} \) reversibly reduced \( I_{K}(f) \) by 58% at 0 mV and 2% at +60 mV (Fig. 8b). At a concentration of 500 \( \mu \)M, \( \text{Zn}^{2+} \) reduced peak \( I_{K}(f) \) by 71 ± 5.4% at 0 mV and by 14 ± 3.4% at +60 mV (n=4) as illustrated for one cell in Fig. 8c. The peak amplitude of \( I_{K}(f) \) was unaffected by 3 \( \mu \)M \( \text{Zn}^{2+} \) (not shown).

\( G_{\text{max}} \) was reduced by 13% with 31 \( \mu \)M \( \text{Zn}^{2+} \) in the external medium (n=3; in one cell it increased 17%) and by approximately 20% when the concentration of \( \text{Zn}^{2+} \) was increased to 62.5 \( \mu \)M (n=5), 125 \( \mu \)M (n=2) or 500 \( \mu \)M (n=4).
Figure 7. The time-dependence of activation and inactivation in 500 μM and 5 mM Cd\textsuperscript{2+}. 

A. The time to half-activation ($t\text{\scriptsize{a}ct}$) is plotted against the test pulse potential for 500 μM Cd\textsuperscript{2+} (diamonds) and its control (open circles) and for 5 mM Cd\textsuperscript{2+} (squares) and its control (filled circles). Data points represent the mean ± s.e.m. of five cells at each concentration of Cd\textsuperscript{2+}. The standard error bars were not included if they were smaller than the symbols.

B. The time to half-inactivation ($t\text{\scriptsize{inact}}$) is plotted against the test pulse potential. Symbols for each concentration of Cd\textsuperscript{2+} and the controls are the same as in A. Data points represent the mean ± s.e.m. of five cells at each concentration of Cd\textsuperscript{2+}. 

C. Superimposed whole-cell currents evoked at 0 mV under control conditions and in the presence of 500 μM Cd\textsuperscript{2+}. The Cd\textsuperscript{2+} trace is scaled x2.25 to allow comparison of the rise times of the current. At 0 mV, $t\text{\scriptsize{a}ct}$ increased from 1.8 ms in control to 4.8 ms in the presence of 500 μM Cd\textsuperscript{2+}. 

Calibration bars apply to the control trace. The zero current level is indicated by the straight line. Cell 140492C.

D. Superimposed whole-cell currents evoked by a test pulse to +20 mV from the holding potential under control conditions and in the presence of 5 mM Cd\textsuperscript{2+}. The current trace in 5 mM Cd\textsuperscript{2+} was scaled x2.71 in order to compare the rise times of the current. At +20 mV, $t\text{\scriptsize{a}ct}$ increased from 1.2 ms in control to 5.1 ms in the presence of 5 mM Cd\textsuperscript{2+}. Cell 1404921.

Solutions: E//E&F [Cd\textsuperscript{2+}]=500 μM///1 and E//F [Cd\textsuperscript{2+}]=5 mM///1.
Table 2. Values listed are the MEAN (±s.e.m.) of the cells examined at each concentration of Cd\textsuperscript{2+}. The number of cells tested "n" is indicated in parentheses in the left column. The data summarized is from four sets of experiments. The control values for each set of experiments are in the row above the treated data. The shift of the activation and steady-state inactivation curves, given as the difference between the mean values of V' obtained in control and those obtained in the Cd\textsuperscript{2+} containing test solutions are summarized in Table 6. N.T. means not tested. Levels of significance for the shifts of the activation and inactivation curves are included in Table 6 (pp 79-80).

\[1 \text{ V'} \text{ represents the membrane potential for half-maximal activation of the current and k represents the slope-factor of the curve fitted to the conductance-voltage relation as described in Section I.}\]

\[2 \text{ V'} \text{ represents the membrane potential at which steady-state half-inactivation of the current is attained and k represents the slope-factor of the curve fitted to the normalized current-voltage relation as described in Section I.}\]

\[3 \text{ n=7 for activation and n=4 for inactivation}\]
| SOLUTION [Cd^{2+}] | ACTIVATION¹ | | | INACTIVATION² | | |
|---------------------|---------------|---------------|---------------|---------------|---------------|
|                     | V' (±S.E.M.) | k (±S.E.M.) | V' (±S.E.M.) | k (±S.E.M.) | |
| Control (n=5)       | -13.7 mV     | 11.5         | -52.4 mV     | -4.1         | |
|                     | (±0.6)       | (±0.9)       | (±1.9)       | (±0.2)       | |
| 500 μM              | +10.9 mV     | 13.2         | -31.3 mV     | -3.4         | |
|                     | (±1.7)       | (±0.4)       | (±2.0)       | (±0.1)       | |
| Control (n=5)       | -12.7 mV     | 15.1         | -52.9 mV     | -4.1         | |
|                     | (±1.1)       | (±0.9)       | (±0.6)       | (±0.2)       | |
| 2 mM                | +17.5 mV     | 9.8          | -19.0 mV     | -4.3         | |
|                     | (±0.6)       | (±0.4)       | (±0.5)       | (±0.1)       | |
| Control (n=7/4)³    | -7.4 mV      | 12.3         | -48.4 mV     | -4.0         | |
|                     | (±2.6)       | (±0.5)       | (±3.6)       | (±0.3)       | |
| 5 mM                | +21.8 mV     | 11.1         | -15.6 mV     | -3.8         | |
|                     | (±1.1)       | (±0.4)       | (±0.3)       | (±0.4)       | |
| Control (n=4)       | -12.9 mV     | 15.7         | N.T.         |               | |
|                     | (±0.6)       | (±0.3)       |               |               | |
| 100 μM              | -4.4 mV      | 11.4         | N.T.         |               | |
|                     | (±0.5)       | (±0.2)       |               |               | |
| 200 μM              | +0.2 mV      | 12.5         | N.T.         |               | |
|                     | (±0.8)       | (±0.4)       |               |               | |
| 400 μM              | +3.7 mV      | 11.8         | N.T.         |               | |
|                     | (±0.8)       | (±0.6)       |               |               | |
| 800 μM              | +8.5 mV      | 12.3         | N.T.         |               | |
|                     | (±1.2)       | (±0.2)       |               |               | |
| 1.6 mM              | +11.4 mV     | 11.3         | N.T.         |               | |
|                     | (±1.3)       | (±0.4)       |               |               | |
1.22 \textbf{Zn}^{2+} \textbf{Causes A Right-Ward Shift Of The Activation Curve}

The apparent voltage-dependent reduction of I_k(f) in the presence of Zn^{2+}, as with Cd^{2+}, is also explained by a Zn^{2+}-induced right-ward shift of the activation curve along the voltage axis. The values for V' and k, (estimated as described in Section I), were determined in each concentration of Zn^{2+} tested and are summarized in Table 3 (pp 55-56). The magnitudes of the shifts of V' with respect to Zn^{2+} concentration are summarized in Table 6 (pp 79-80).

The filled symbols in Figs. 9a, 9b & 9d represent the mean (±s.e.m.) normalized chord conductance at each potential in the presence of 62.5 \mu M, 125 \mu M and 500 \mu M Zn^{2+}, respectively. The data points (filled circles and filled diamonds) in Fig. 9c represent the normalized chord conductance calculated at each potential in the presence of 250 \mu M Zn^{2+} for one cell only.

At a concentration of 31 \mu M, Zn^{2+} did not significantly (p>0.01) shift the activation curve nor did 3 \mu M Zn^{2+} shift the activation curve (see Tables 3 and 6). A [Zn^{2+}]_o of 62.5 \mu M shifted V' by +12.8 ± 2.8 mV (p<0.01) from -12.7 ± 1.9 mV in control (filled circles) to +0.1 ± 0.9 mV (filled diamonds, Fig. 9a; n=4). Increasing the [Zn^{2+}]_o to 125 \mu M shifted V' an average of +20.9 mV from -14.7 ± 0.9 mV in control (filled circles) to +6.2 ± 0.5 mV (filled diamonds, Fig. 9b; n=2). For the one cell examined at 250 \mu M Zn^{2+}, V' was shifted by +27.5 mV, from -17.7 mV (filled circles) in control to +9.8 mV (filled diamonds, Fig. 9c). The Zn^{2+} effect on the activation curve appeared to saturate near 250 \mu M since increasing [Zn^{2+}]_o to 500 \mu M shifted V' by +26.1 ± 4.3 mV (p<0.01), from a control value of -11.0 ± 2.0 mV (filled circles) to +15.1 ± 2.3 mV (n=4; filled diamonds; Fig. 9d).

\textbf{Zn}^{2+} did not alter the slope-factor of the conductance-voltage relation at any of the concentrations tested (see Table 3).
Figure 8. Concentration-dependent effects of Zn\(^{2+}\) on the current-voltage relation of I\(_K\)(f).

A. Normalized current-voltage relations for I\(_K\)(f) under control conditions (open circles) and in the presence of 31 µM (squares), 62.5 µM (triangles) and 125 µM Zn\(^{2+}\) (diamonds). Cell 271191B. The effects of Zn\(^{2+}\) on I\(_K\)(f) were completely reversible (filled circles).

B. The normalized current-voltage relation for I\(_K\)(f) in one cell (271191A) in 250 µM Zn\(^{2+}\) (diamonds). The current was normalized against maximum I\(_K\)(f) in control. The normalized current was plotted against the test pulse potential. A Zn\(^{2+}\) concentration of 250 µM shifted the threshold for activation from the control (open circles) value of -40 mV to -20 mV. Consistent with this shift the current evoked at each test potential up to +60 mV was reduced in the presence of 250 µM Zn\(^{2+}\). At test potentials greater than +60 mV the current-voltage relations overlap. The effects of 250 µM Zn\(^{2+}\) on I\(_K\)(f) were reversible (filled circles).

C. The normalized current-voltage relation elicited in the same manner as in A and B but in the presence of 500 µM Zn\(^{2+}\) (diamonds). The threshold for activation shifted from the control (open circles) value of -40 mV to -10 mV. Commensurate with this shift, the current evoked at each test potential in the presence of 500 µM Zn\(^{2+}\) was reduced. The effects of 500 µM Zn\(^{2+}\) were partially reversible (filled circles).

Solutions: G//H [Zn\(^{2+}\)]=31 to 500 µM///1.
1.23 Concentration Dependence For Zn$^{2+}$-Induced Shifts Of V'

The concentration dependence of the Zn$^{2+}$-induced shift of V' is illustrated in Fig. 10. A least squares fit of the Michaelis-Menton equation to the data showed that the Zn$^{2+}$-induced shift of V' saturated at +34 mV with a $K_M$ of 92 µM.

1.24 Zn$^{2+}$ Increases The 50% Rise Time Of $I_K(t)$

At all concentrations examined, with the exception of 3 µM, Zn$^{2+}$ increased the rise time of the current especially at membrane potentials close to the threshold for activation. Half-activation times were examined at 500 µM Zn$^{2+}$ to allow qualitative comparisons to the results observed with Cd$^{2+}$.

The 50% rise times, determined at four potentials, are summarized in Fig. 11a. At 0 mV, there was a 4.7-fold increase ($n=4$) in $t_{50\text{act}}$ from 2.1 ± 0.2 ms in control (circles; Fig. 11a) to 9.7 ± 0.9 ms in 500 µM Zn$^{2+}$ (diamonds; Fig. 11a). This effect of Zn$^{2+}$ on the activation kinetics is evident in the current traces in Fig. 11c where the current trace recorded in 500 µM Zn$^{2+}$ at 0 mV was scaled up 5.35-fold, in order to allow a comparison of the rising phase of the current. As the potential was stepped to more depolarized levels the slowing of the activation kinetics by Zn$^{2+}$ was less pronounced as indicated by the graph of Fig. 11a and by the current traces of Fig. 11d at +60 mV. At +60 mV, there was a 2-fold increase in $t_{50\text{act}}$ ($n=4$) from 0.9 ± 0.1 ms in control (circles; Fig. 11a) to 2.0 ± 0.1 ms in 500 µM Zn$^{2+}$ (diamonds; Fig. 11a).

1.25 Zn$^{2+}$ Causes A Right-Ward Shift Of The Steady-State Inactivation Curve

The effect of Zn$^{2+}$ on steady-state inactivation was examined at 3-500 µM [Zn$^{2+}$] and the values for V' and k, (estimated as described in Section I) are summarized in Table 3. The magnitudes of Zn$^{2+}$-induced shifts of V' for the h$_a$ curve are summarized in Table
Figure 9. Control and treated activation and inactivation curves in Zn\textsuperscript{2+} concentrations of 62.5 μM (A), 125 μM (B), 250 μM (C), and 500 μM (D). A. The data points represent the mean ± s.e.m. of four cells. For the activation curve, control V' was -12.7 ± 1.9 mV and the slope-factor (k) was 13.2 ± 0.6 mV (filled circles) versus V' = -0.1 ± 0.9 mV and k = 12.8 ± 0.6 mV (filled diamonds) in 62.5 μM Zn\textsuperscript{2+}. For the inactivation curves, V' = -54.0 ± 0.3 mV and k = -4.7 ± 0.1 mV in control (open circles) versus V' = -39.2 ± 0.9 mV and k = -5.0 ± 0.3 mV (open diamonds). B. The data points represent the mean ± s.e.m. of two cells. V' for activation shifted from -14.7 ± 0.9 mV in control (filled circles) to +6.2 ± 0.5 mV in medium containing 125 μM Zn\textsuperscript{2+} (filled diamonds). The respective slope-factors were 12.1 ± 1.8 mV and 13.9 ± 0.1 mV. The inactivation curves were best fit with V' = -56.5 ± 2.5 mV and k = -4.7 ± 0.2 mV in control (open circles) versus V' = -39.2 ± 0.9 mV and k = -5.0 ± 0.0 mV in 125 μM Zn\textsuperscript{2+} (open diamonds). C. The activation and inactivation curves in 250 μM Zn\textsuperscript{2+} were determined for one cell (271191A). For the activation curve, control V' and k were -17.7 mV and 11.0 mV, respectively (filled circles) versus V' = +9.8 mV and k = 12.3 mV (filled diamonds). For the inactivation curve, in control (open circles), V' = -54.9 mV and k = -4.2 mv versus V' = -28.8 mV and k = -5.1 mV (open diamonds). D. The data points represent the mean ± s.e.m. of four cells in 500 μM Zn\textsuperscript{2+}. For control activation (filled circles), V' = -11.0 ± 2.0 mV and k = 12.5 mV versus V' = +15.1 ± 2.3 mV and k = 12.4 mV (filled diamonds). Steady-state half-inactivation shifted from control (open circles) V' = -53.1 ± 1.2 mV to V' = -27.2 ± 1.4 mV (open diamonds). The respective slope-factors were k = -4.5 ± 0.1 mV and k = -5.1 ± 0.3 mV.
6. The data points (open symbols) in Fig. 9a, b, d represent the mean normalized current ± s.e.m. at each potential in the presence of 62.5 μM, 125 μM and 500 μM Zn^{2+}, respectively. The data points (open circles and open diamonds) in Fig. 9c represent the normalized current calculated at each potential in the presence of 250 μM Zn^{2+} for one cell only.

Bath application of 3 μM Zn^{2+} had no effect on the h_{m} curve. However, 31 μM Zn^{2+} which did not significantly (p>0.01) shift the activation curve did shift V' of the h_{m} curve by +8.5 ± 3.3 mV (see Table 3). Shifts of +14.9 ± 1.2 mV (n=4; p<0.01) and +19.4 mV (n=2) for V' of the h_{m} curve were obtained with 62.5 μM Zn^{2+} and 125 μM Zn^{2+}, respectively (open symbols; Figs. 9a & 9b, respectively). The Zn^{2+}-induced shift of V' for inactivation also appeared to saturate near 250 μM Zn^{2+}. For the one cell examined, 250 μM Zn^{2+} shifted V' by +26 mV from -54.9 mV in control (open circles; Fig. 9c) to -28.8 mV (open diamonds; Fig. 9c). When the [Zn^{2+}]_{o} was increased to 500 μM there was no further increase in the shift of V'. At a concentration of 500 μM, Zn^{2+} also shifted V' by +26.0 ± 2.5 mV (n=4; p<0.01) from -53.1 ± 1.2 mV in control (open circles; Fig. 9d) to -27.2 ± 1.4 mV (open diamonds; Fig. 9d).

1.26 Zn^{2+} Increases The Time To Half-Inactivation

Time to half-inactivation increased approximately 1.4-fold at all potentials in the presence of 500 μM Zn^{2+}. At 0 mV, t_{1/2 \text{inact}} increased from 27.9 ± 0.7 ms in control (circles, Fig. 11b) to 42.7 ± 5.3 ms in 500 μM Zn^{2+} (diamonds, Fig. 11b) and at +60 mV t_{1/2 \text{inact}} increased from 40.2 ± 0.2 ms in control to 54.4 ± 1.6 ms in 500 μM Zn^{2+}.
Figure 10. The concentration dependence of the Zn$^{2+}$-induced shift of the half-activation potential for $I_K(f)$. Data points represent the mean shift ± s.e.m. unless there was insufficient data to calculate s.e.m. in which case no error bar is included; the bracketed number indicates the value for "n". Shifts of the activation curve by Zn$^{2+}$ (as summarized in Table 6) are plotted as a function of Zn$^{2+}$ concentration. The line represents a solution to the Michaelis-Menton equation in which $S_{\text{max}}$ was +34 mV and the $K_M$ was 92 μM.
Voltage shift (mV) vs. Zn$^{2+}$ concentration (µM)
Figure 11. The time-dependence of activation and inactivation in the presence of 500 μM Zn\(^{2+}\). (A) The time to half-activation (\(t_{\text{\(\text{act}\)}}\)) (mean ± s.e.m., n=4) is plotted against the test pulse potential for currents evoked in 500 μM Zn\(^{2+}\) (diamonds) or in control medium (open circles). B. The time for half-inactivation (\(t_{\text{\(\text{inact}\)}}\)) is plotted against the membrane potential during the test pulse. Symbols are the same as in A. Data points represent the mean ± s.e.m. (n=4). C. Superimposed whole-cell currents elicited by a test pulse to 0 mV from the holding potential under control conditions and in 500 μM Zn\(^{2+}\). The Zn\(^{2+}\) trace is scaled 5.35-fold to allow comparison of the rise times of the current. At this membrane potential, \(t_{\text{\(\text{act}\)}}\) increased from 2.0 ms in control to 9.8 ms in the presence of 500 μM Zn\(^{2+}\). Calibration bars apply to the control trace. Cell 201191D. D. Superimposed whole-cell currents evoked by a test pulse to +60 mV from the holding potential for the same cell as in C, in control medium and modified control containing 500 μM Zn\(^{2+}\). The current trace in 500 μM Cd\(^{2+}\) was scaled 1.25-fold in order to compare the rise times of the current under the control and test conditions. The effect of Zn\(^{2+}\) on the rise time of \(I_K(f)\) is less pronounced at more depolarized potentials. At +60 mV, \(t_{\text{\(\text{act}\)}}\) increased from 0.87 ms in control to 1.87 ms in the presence of 500 μM Zn\(^{2+}\). Cell 201191D. Solutions: G//H \([\text{Zn}^{2+}] = 500 \mu M//1\).
Table 3. Values summarized are the MEAN (±s.e.m.). The number of cells "n" examined is indicated in parentheses below each solution. The control values for each set of experiments precede the values for the test conditions. The shift of the activation and steady-state inactivation curves, given as the difference between the mean values of V' in the control and Zn²⁺ containing solutions, respectively, are summarized in Table 6. Levels of significance for the shifts of the activation and inactivation curves are included in Table 6 (pp 79-80).

¹ n = 4 for activation and n = 5 for inactivation
TABLE 3. THE VALUES OF V' AND k CALCULATED FOR THE ACTIVATION AND INACTIVATION OF Ik(f) IN THE PRESENCE OF Zn²⁺

<table>
<thead>
<tr>
<th>SOLUTION [Zn²⁺]</th>
<th>ACTIVATION</th>
<th>INACTIVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V' (±S.E.M.)</td>
<td>k (±S.E.M.)</td>
</tr>
<tr>
<td>Control (n=2)</td>
<td>-18.1 mV (±4.7)</td>
<td>12.2 (±4.0)</td>
</tr>
<tr>
<td>3 µM</td>
<td>-20.2 mV (±1.3)</td>
<td>14.7 (±1.1)</td>
</tr>
<tr>
<td>Control (n=4)</td>
<td>-14.5 mV (±4.2)</td>
<td>11.8 (±1.7)</td>
</tr>
<tr>
<td>31 µM</td>
<td>-7.4 mV (±5.3)</td>
<td>11.9 (±1.8)</td>
</tr>
<tr>
<td>Control (n=4/5)</td>
<td>-12.7 mV (±1.9)</td>
<td>13.2 (±0.6)</td>
</tr>
<tr>
<td>62.5 µM</td>
<td>+0.1 mV (±0.9)</td>
<td>12.8 (±0.6)</td>
</tr>
<tr>
<td>Control (n=2)</td>
<td>-14.7 mV (±0.9)</td>
<td>12.1 (±1.8)</td>
</tr>
<tr>
<td>125 µM</td>
<td>+6.2 mV (±0.5)</td>
<td>13.9 (±0.1)</td>
</tr>
<tr>
<td>Control (n=1)</td>
<td>-17.7 mV (±0.9)</td>
<td>11.0 (±1.0)</td>
</tr>
<tr>
<td>250 µM</td>
<td>+9.8 mV (±0.3)</td>
<td>12.3 (±0.3)</td>
</tr>
<tr>
<td>Control (n=4)</td>
<td>-11.0 mV (±2.0)</td>
<td>12.5 (±0.7)</td>
</tr>
<tr>
<td>500 µM</td>
<td>+15.1 mV (±2.3)</td>
<td>12.4 (±0.3)</td>
</tr>
</tbody>
</table>
2. The Effects Of Alkaline Earth Metal Ions On $I_K(f)$

Divalent cations from Group IIB, though less potent than the transition metal ions, have been reported to exert qualitatively similar effects on the behaviour of voltage-gated ion channels (McLaughlin et al., 1971; Hahin & Campbell, 1983; Cukierman & Krueger, 1990). Of the alkaline earth metal ions, $Ca^{2+}$ is reported to have the greatest and $Mg^{2+}$ the smallest effect. The following results indicate that the same pattern persists for the $I_K(f)$ of melanotrophs.

2.1 Calcium

$Ca^{2+}$ is postulated to play a role in the regulation of $Na^+$ channel gating (Armstrong & Cota, 1991), the activation of some TOC-like currents (Zbicz & Weight, 1985), and the maintenance of $K^+$ channel integrity (Armstrong & López-Barneo, 1987). External $Ca^{2+}$ also causes a voltage-dependent block of $Na^+$ channels (Woodhull, 1973) in addition to producing a non-specific screening of surface charges (Frankenhaeuser & Hodgkin, 1957). Thus, it was of interest to determine whether $Ca^{2+}$ might exert similar effects on the behaviour of $I_K(f)$.

2.11 Changes in $[Ca^{2+}]_o$ Reduce $I_K(f)$

The reduction of $I_K(f)$ in external medium containing zero $Ca^{2+}$ is illustrated in the current-voltage relation for one cell in Fig. 12a (note that the solution is nominally $Ca^{2+}$-free as a $Ca^{2+}$ buffer was not included - the contaminating concentration of $Ca^{2+}$ could be as high as 2.5 $\mu$M). In the four cells examined, zero $Ca^{2+}$ reduced $I_K(f)$ by $18 \pm 2.1\%$ at 0 mV and $21 \pm 3.0\%$ at +60 mV and decreased $G_{max}$ by $21 \pm 3.0\%$. This decline of $I_K(f)$ probably reflects a left-ward shift of the $h_o$ curve. During the protocol to determine the current-voltage relation the holding potential was -80 mV; due to the left-ward shift of the $h_o$ curve in zero $Ca^{2+}$ approximately 20% of the current is inactivated at this potential which is consistent with the reduction in peak $I_K(f)$ and $G_{max}$ observed.
Increasing $[\text{Ca}^{2+}]_o$ to 5 mM, 10 mM and 20 mM also reduced the peak amplitude of $I_{K}(f)$ as shown for one cell in Fig. 12b. The peak amplitude of $I_{K}(f)$ and the chord conductance at +60 mV for this cell were reduced by 5%, 7% and 21% in 5, 10 and 20 mM Ca$^{2+}$, respectively. For purposes of comparison, the chord conductance was determined at +60 mV in all concentrations of Ca$^{2+}$ tested. In control medium, $G_{\text{max}}$ is reached at +60 mV, however, increasing $[\text{Ca}^{2+}]_o$ shifts the conductance-voltage relation right-ward along the voltage axis and it is unlikely that $G_{\text{max}}$ was attained at +60 mV in the test conditions. Therefore, an unequivocal conclusion regarding the effect of increased $[\text{Ca}^{2+}]_o$ on $G_{\text{max}}$ cannot be made.

### 2.12 Changes in $[\text{Ca}^{2+}]_o$ Shift V’ For The Activation Curve

The values for V’ and k, (estimated as described in Section I), determined in 0-20 mM Ca$^{2+}$ are summarized in Table 4 (pp 69-70). The magnitudes of the shifts of V’ with respect to the external Ca$^{2+}$ concentration are summarized in Table 6 (pp 79-80).

The conductance-voltage relation shifted left-ward along the voltage axis in zero Ca$^{2+}$ as illustrated for a representative cell in Fig. 13a (filled symbols). For the four cells examined, removing Ca$^{2+}$ from the external medium shifted half-activation by -11.7 ± 2.7 mV from -15.2 ± 1.6 mV in control to -26.9 ± 1.1 mV. In zero Ca$^{2+}$ the slope-factor (k) of the conductance-voltage relation increased from 13.6 ± 0.9 mV to 22.0 ± 1.3 mV (see Table 4) which reflects a decrease in the voltage-sensitivity of activation.

Increasing $[\text{Ca}^{2+}]_o$, from the control concentration of 2 mM, caused a concentration-dependent right-ward shift of the activation curve (Fig. 13b). A $[\text{Ca}^{2+}]_o$ of 5 mM shifted V’ $+2.5 \pm 1.8$ mV from -10.6 ± 1.7 in control to -8.1 ± 0.0 (n=2). Raising the Ca$^{2+}$ concentration to 10 mM shifted V’ by $+8.4 \pm 2.3$ mV from the control value above to -2.2 ± 0.6 mV (n=2). A Ca$^{2+}$ concentration of 20 mM shifted V’ $+12.4 \pm 2.2$ mV from the control value of -10.6 ± 1.7 to $+1.8 \pm 0.5$ mV (n=3).
**Figure 12.** Current-voltage relations for normalized $I_K(f)$ in response to reduced or increased concentrations of external Ca$^{2+}$.  

**A.** The peak amplitude of $I_K(f)$ was reduced in zero Ca$^{2+}$ (diamonds) relative to the control responses (circles) when 2 mM Ca$^{2+}$ was present. At 0 mV, $I_K(f)$ was reduced 15.7% in zero Ca$^{2+}$ relative to the control and at +60 mV $I_K(f)$ was reduced by 24.1% relative to control. Solutions: B//I///3. Cell 260891D.

**B.** As the external concentration of Ca$^{2+}$ was increased from the control concentration of 2 mM (circles) to 5 mM (diamonds), 10 mM (squares) and 20 mM Ca$^{2+}$ (triangles) there was a 5%, 7%, and 21% decrease, respectively, in the normalized peak amplitude of $I_K(f)$. Solutions: B//J [Ca$^{2+}]=5-20$ mM///3. Cell 280891H.
Increasing [Ca\textsuperscript{2+}]\textsubscript{o} decreased the slope-factor (k) of the conductance-voltage relation from 14.7 ± 0.4 mV in control to 11.8 ± 0.3 mV in 5 mM Ca\textsuperscript{2+}, 10.7 ± 0.3 mV in 10 mM Ca\textsuperscript{2+} and 9.5 ± 0.5 mV in 20 mM Ca\textsuperscript{2+}.

2.13 Concentration Dependence For Ca\textsuperscript{2+-}Induced Shifts of V'

The curve fitted to the data points of Fig. 14 indicates that the maximal Ca\textsuperscript{2+}-induced shift of V' (from the control concentration of 2 mM) was +15.6 mV and that the half-maximal shift occurred at a [Ca\textsuperscript{2+}]\textsubscript{o} of 3.4 mM. The K\textsubscript{m} for the shift of V' is approximately one order of magnitude greater than that calculated for either Cd\textsuperscript{2+} or Zn\textsuperscript{2+} and the maximum depolarized shift is approximately half that possible with the two transition metal ions.

2.14 Increasing [Ca\textsuperscript{2+}]\textsubscript{o} Increases t\textsubscript{\text{h,act}} For I\textsubscript{K}(f)

Increasing [Ca\textsuperscript{2+}]\textsubscript{o} increased the time to half-activation at potentials close to the threshold for activation (Fig. 15a). The increase in the rise time of I\textsubscript{K}(f) was most noticeable in 20 mM Ca\textsuperscript{2+} where t\textsubscript{\text{h,act}} increased approximately 2-fold at 0 mV. At +60 mV, there was no measurable difference in the rise time of the current at any concentration of Ca\textsuperscript{2+} examined.

2.15 Changes in [Ca\textsuperscript{2+}]\textsubscript{o} Shift V' For The Inactivation Curve

The effect of Ca\textsuperscript{2+} on steady-state inactivation was examined and the values for V' and k (estimated as described in Section I) are summarized in Table 4.

In zero Ca\textsuperscript{2+} there was a left-ward shift of the inactivation curve (Fig. 13a, open symbols). V' was shifted -19.5 ± 5.2 mV (p<0.01) from a control value of -54.1 ± 1.3 mV (open circles) to -73.5 ± 3.9 mV (open diamonds; n=4).
Figure 13. The effect of changes of $[\text{Ca}^{2+}]_o$ on the activation and steady-state inactivation curves for $I_{\text{k}(f)}$. A. The normalized chord conductance is plotted against the test pulse potential and the activation curve is a solution to the Boltzman equation where $V' = -12.4$ mV and the slope-factor = 15.0 mV for control (filled circles) versus $V' = -25.6$ mV and a slope-factor = 23.4 mV in zero Ca$^{2+}$ (filled diamonds). For steady-state inactivation, the curve is a solution to the Boltzman equation where $V' = -51.1$ mV and the slope-factor $(k)$ = -4.0 mV for control (open circles) whereas in zero Ca$^{2+}$ $V' = -67.8$ mV and $k$ = -5.2 mV in zero Ca$^{2+}$ (open diamonds). Solutions: B//I///3. Cell 260891D. B. The details of these activation and steady-state inactivation curves are given in the text. Activation: control (filled circles); 5 mM Ca$^{2+}$ (filled diamonds); 10 mM Ca$^{2+}$ (filled squares); 20 mM Ca$^{2+}$ (filled triangles). Inactivation: control (open circles); 5 mM Ca$^{2+}$ (open diamonds); 10 mM Ca$^{2+}$ (open squares); 20 mM Ca$^{2+}$ (open triangles). Solutions: B//J $[\text{Ca}^{2+}]_o = 5$-20 mM///3. Cell 280891H.
Figure 14. Concentration-response for the effects of Ca\(^{2+}\) on the shift of the half-activation potential (V'). Shifts of V' as summarized in Table 6 are plotted against external Ca\(^{2+}\) concentration. Data points represent the mean ± s.e.m.; "n" is indicated in brackets. The fitted curve is a solution to the Michaelis-Menton equation where the maximal shift is +15.6 mV and the K\(_M\) for the half-maximal shift is 3.4 mM.
When \([\text{Ca}^{2+}]_0\) was raised there was a concentration-dependent right-ward shift of the inactivation curve along the voltage axis (Fig. 13b). Increasing \([\text{Ca}^{2+}]_0\) to 5 mM shifted \(V'\) by approximately +6.3 mV from -52.7 ± 1.5 mV in control (open circles) to -46.5 ± 0.4 mV (open diamonds; \(n=2\)). After changing to 10 mM \(\text{Ca}^{2+}\), \(V'\) shifted approximately +11.6 mV from the same control value to -41.1 ± 0.4 mV (open diamonds; \(n=2\)). Finally, a 20 mM concentration of \(\text{Ca}^{2+}\) shifted \(V'\) by approximately +18.8 mV (\(n=5\); \(p<0.01\)) from the control value of -52.7 ± 1.5 mV (open circles) to -33.9 ± 1.7 mV (open triangles). The shifts observed with \(\text{Ca}^{2+}\) in the millimolar range were not as great as those observed with a thousand-fold lower concentration of either \(\text{Cd}^{2+}\) or \(\text{Zn}^{2+}\).

When the concentration of \(\text{Ca}^{2+}\) was raised or lowered the steady-state inactivation curves shifted in a parallel fashion and there was no change in the slope (\(k\)) of the curves (see Table 4).

2.16 Influence Of Changing \([\text{Ca}^{2+}]_0\) On \(t_{\text{inact}}\) For \(I_{K}(f)\)

The time to half-inactivation did not change from control values with 5 mM or 10 mM external \(\text{Ca}^{2+}\). A 20 mM concentration of \(\text{Ca}^{2+}\) increased \(t_{\text{inact}}\) approximately 1.2-fold at 0 mV and 1.0-fold at +60 mV in the two cells examined (Fig. 15b).

2.2 Magnesium

Among the divalent cations, \(\text{Mg}^{2+}\) is one of the least potent in causing right-ward shifts of the activation and inactivation curves of the \(\text{Na}^+\) and delayed rectifier \(\text{K}^+\) channel (Blaustein & Goldman, 1968; Hille et al., 1975). The effect of \(\text{Mg}^{2+}\) was examined to determine if this finding was consistent with respect to \(I_{K}(f)\).
**Figure 15.** The effect of increased $[\text{Ca}^{2+}]_o$ on the activation and inactivation kinetics of $I_K(f)$. A. The half-activation times ($t_{\text{act}}^A$) in control (circles) and medium containing 5 mM (diamonds), 10 mM (squares) or 20 mM (triangles) $\text{Ca}^{2+}$. Each point represents the mean for two cells. At -20 mV, $t_{\text{act}}^A$ increased approximately 1.3-fold, 1.9-fold, and 3.2-fold, respectively, in the presence of 5, 10 and 20 mM $\text{Ca}^{2+}$ relative to a $t_{\text{act}}^A$ of 3.32 ms in control at -20 mV. At +60 mV, $t_{\text{act}}^A$ was increased maximally (approximately 1.5-fold) in the presence of 20 mM $\text{Ca}^{2+}$. B. The half-inactivation time for $I_K(f)$ is plotted as in A. There was no difference in $t_{\text{inact}}$ between the control values and the values with 5 and 10 mM $\text{Ca}^{2+}$ present. The shift in $t_{\text{inact}}$ with 20 mM $\text{Ca}^{2+}$ in the bath is described in the text.

**Solutions:** B//J $[\text{Ca}^{2+}]=5$-20 mM///3.
Table 4. Values summarized are the MEAN (±s.e.m.). The number of cells "n" examined is indicated in parentheses below each solution. The shift of the activation and steady-state inactivation curves, given as the difference between the mean values of V' in the control and test solutions, respectively, are summarized in Table 6. Levels of significance for the shifts of the activation and inactivation curves are included in Table 6 (pp 79-80).

1 As described in the methods, the solution identified as 0 mM Ca\(^{2+}\) can only be considered nominally Ca\(^{2+}\) free as a Ca\(^{2+}\) buffer was not included in the solution.

2 The conductance-voltage relation was completed for n = 2 cells and steady-state inactivation was completed for n = 4 cells.

3 The conductance-voltage relation was examined for 3 cells and steady-state inactivation was determined for 4 cells.

† The slope of the activation curve in zero Ca\(^{2+}\) is significantly greater than in control (p < 0.01). Significance was determined using a paired "t"-test with p = 0.01.

‡ The slope of the inactivation curve in zero Ca\(^{2+}\) is not significantly greater than in control with (p < 0.03). The significance level was set at p = 0.01 to account for the small sample size. However, as the change would be significant with p = 0.05 the increase in the slope of the inactivation curve warrants closer examination.
TABLE 4. THE VALUES OF $V'$ AND $k$ FOR THE ACTIVATION AND INACTIVATION OF $I_K(f)$ IN THE PRESENCE AND ABSENCE OF $Ca^{2+}$

<table>
<thead>
<tr>
<th>SOLUTION$^1$ $[Ca^{2+}]$</th>
<th>ACTIVATION</th>
<th></th>
<th></th>
<th>INACTIVATION</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V' (±S.E.M.)</td>
<td>k (±S.E.M.)</td>
<td>V' (±S.E.M.)</td>
<td>k (±S.E.M.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=4)</td>
<td>-15.2 mV (±1.6)</td>
<td>13.6 (±0.9)</td>
<td>-54.1 mV (±1.3)</td>
<td>-3.8 (±0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM (n=4)</td>
<td>-26.9 mV (±1.1)</td>
<td>22.0 (±1.3)</td>
<td>-73.5 mV (±3.9)</td>
<td>-5.4 (±0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (2 mM) (n=3)</td>
<td>-10.6 mV (±1.7)</td>
<td>14.7 (±0.4)</td>
<td>-52.7 mV (±1.5)</td>
<td>-4.3 (±0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM (n=2\4)</td>
<td>-8.1 mV (±0.0)</td>
<td>11.8 (±0.3)</td>
<td>-45.7 mV (±1.8)</td>
<td>-4.1 (±0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM (n=2\4)</td>
<td>-2.2 mV (±0.6)</td>
<td>10.7 (±0.3)</td>
<td>-40.5 mV (±1.8)</td>
<td>-3.8 (±0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mM (n=3\4)</td>
<td>1.8 mV (±0.5)</td>
<td>9.5 (±0.5)</td>
<td>-34.5 mV (±2.1)</td>
<td>-3.5 (±0.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.21 Voltage- and Time-Dependence of Activation For $I_K(f)$
In The Presence of Mg\(^{2+}\)

Mg\(^{2+}\) at a concentration of 10 mM had very little effect on the current-voltage relation for $I_K(f)$. Consistent with these observations, $V'$ and $k$ (estimated as in Section I) for the conductance-voltage relation were unchanged from the control values (Fig. 16a).

In 40 mM Mg\(^{2+}\) the threshold for activation shifted from -40 mV to -30 mV and at potentials below +60 mV the peak amplitude of $I_K(f)$ was reduced. At potentials above +60 mV, 40 mM Mg\(^{2+}\) had no effect on the peak amplitude of $I_K(f)$. These results are consistent with the right-ward shift of the activation curve caused by 40 mM Mg\(^{2+}\): the half-activation potential shifted by $2.7 \pm 3.9$ mV ($p<0.01$) from $-13.8 \pm 1.9$ mV in control (Fig. 16b, filled circles) to $-11.2 \pm 2.0$ mV in 40 mM Mg\(^{2+}\) (Fig. 16b, filled diamonds; $n=4$). A 40 mM concentration of Mg\(^{2+}\) shifted the activation and inactivation curves along the voltage axis to approximately the same degree as 20 mM Ca\(^{2+}\), 200 $\mu$M Cd\(^{2+}\) or 62.5 $\mu$M Zn\(^{2+}\) (see Table 6). The slope-factor ($k$) of the conductance-voltage relation was not altered in the presence of 40 mM Mg\(^{2+}\) (see Table 5 - pp 77-78).

As illustrated in Fig. 17a, 40 mM Mg\(^{2+}\) increased $t_{\text{act}}$ in a manner similar to that observed with the other divalent cations. The action of 40 mM Mg\(^{2+}\) to slow the activation of $I_K(f)$ is illustrated in the current traces of Fig. 17b in which the treated current has been scaled and superimposed on the control current. At 0 mV, 40 mM Mg\(^{2+}\) increased $t_{\text{act}}$ 1.8-fold from $1.4 \pm 0.1$ ms to $2.5 \pm 0.2$ ms ($n=4$). As the membrane was stepped to more depolarized levels there was less of an effect on the rise time by Mg\(^{2+}\) consistent with the results obtained with Cd\(^{2+}\), Zn\(^{2+}\) and Ca\(^{2+}\).

2.22 Mg\(^{2+}\) Shifts V' For Steady-State Inactivation

The effects of Mg\(^{2+}\) on steady-state inactivation were comparable to its effects on activation. Thus, a concentration of 10 mM Mg\(^{2+}\) had very little effect on steady-state
Figure 16. The effects of 10 mM (A) and 40 mM (B) Mg$^{2+}$ on the activation and inactivation curves for $I_K(f)$. A. In 10 mM Mg$^{2+}$, $V'$ shifted from -12.4 mV in control (filled circles) to -13.2 mV in 10 mM Mg$^{2+}$ (filled diamonds), and the slope-factor decreased from a control value of 13.3 mV to 10.9 mV. The inactivation curve shifted from a half-inactivation potential of -55.0 mV with a slope-factor of -3.9 mV in control (open circles) to a half-inactivation of -50.8 mV with a slope-factor of -4.0 mV (open diamonds). Circles inset with crosses represent the recovery responses. Cell 241091B. Solutions: K//L&M $[Mg^{2+}]_o=10$ mM///1. B. A 40 mM concentration of Mg$^{2+}$ shifted the activation and steady-state inactivation curves right-ward along the voltage axis. The activation curve for this cell shifted from $V' = -16.5$ mV with a slope-factor of 11.6 mV in control (filled circles) to $V' = -1.0$ mV with a slope-factor of 12.3 mV in 40 mM Mg$^{2+}$ (filled diamonds). The inactivation curve shifted from $V' = -59.8$ mV with a slope-factor of -4.4 mV in control (open circles) to $V' = -45.9$ mV with a slope-factor of -5.1 mV in 40 mM Mg$^{2+}$ (open diamonds). Cell 171091D. Solutions: K//M $[Mg^{2+}]=40$ mM///1.
inactivation as illustrated for one cell in Fig. 16a (open circles and diamonds). The shift of the $h_o$ curve caused by 40 mM Mg$^{2+}$ was equivalent to the shift of the conductance-voltage relation and is illustrated for one cell in Fig. 16b. $V'$ shifted by $+17.2 \pm 3.8$ mV from $-60.4 \pm 2.3$ mV in control to $-43.2 \pm 1.5$ mV in 40 mM Mg$^{2+}$ ($n=4$).

The half-inactivation time was unaffected by either 10 mM or 40 mM Mg$^{2+}$ (not shown).
Figure 17. The time-dependence of activation in the presence of 40 mM Mg$^{2+}$. A. The time to half-activation is plotted against the test pulse potential. Data points represent the mean ± s.e.m. for 3 cells recorded in solutions: K$^{+}$/Mg$^{2+}$=40 mM///1. A 40 mM concentration of Mg$^{2+}$ (diamonds) increased the time to half-activation compared to the control values (circles) at all potentials. See text for details. B. Superimposed whole-cell currents evoked at 0 mV. The current trace obtained in 40 mM Mg$^{2+}$ was scaled x1.53 in order that the rise times of the currents could be compared. Mg$^{2+}$, even at a concentration of 40 mM, was less potent than any of the other divalents tested in slowing the rise of $I_k(f)$. At 0 mV, $t_{\%act}$ increased from 1.4 ± 0.1 ms in control to 2.5 ± 0.2 ms in 40 mM Mg$^{2+}$. 
Table 5. Values summarized are the MEAN (±s.e.m.). The number of cells examined "n" is indicated in parentheses below each solution. Experiments using 10 mM Mg\(^{2+}\) were run separately from those with 40 mM Mg\(^{2+}\). The control values for each experiment are summarized in the row above the treated values. The shifts of the activation and steady-state inactivation curves caused by Mg\(^{2+}\), given as the difference between the mean values of \(V'\) in the control and Mg\(^{2+}\) containing solutions, respectively, are summarized in Table 6. Levels of significance for the shifts of the activation and inactivation curves are included in Table 6 (pp 79-80).
### Table 5. The Values of V' and k Calculated for the Activation and Inactivation of $I_\kappa(f)$ in the Presence of Mg$^{2+}$

<table>
<thead>
<tr>
<th>SOLUTION [Mg$^{2+}$]</th>
<th>ACTIVATION</th>
<th>INACTIVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V' (±S.E.M.)</td>
<td>k (±S.E.M.)</td>
</tr>
<tr>
<td>Control (1 Mg$^{2+}$)</td>
<td>-15.2 mV (±2.1)</td>
<td>11.3 (±1.0)</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM (n=6)</td>
<td>-15.9 mV (±2.5)</td>
<td>11.8 (±0.9)</td>
</tr>
<tr>
<td>Control (1 Mg$^{2+}$)</td>
<td>-13.8 mV (±1.9)</td>
<td>12.8 (±0.9)</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 mM (n=4)</td>
<td>-1.2 mV (±2.0)</td>
<td>12.1 (±1.7)</td>
</tr>
</tbody>
</table>
Table 6.  A summary of the results presented in Tables 2, 3, 4 and 5. The voltage shifts were determined by the difference between the mean half-activation or half-inactivation potentials in the control medium, and the medium containing the divalent cation indicated. The number of cells from which the mean (± s.e.m.) was determined is indicated to the right in parenthesis. N.T. means not tested.

1 Significance of the shifts was not examined for this series of experiments since the steady-state inactivation was not tested and complete data was obtained at three other concentrations of Cd$^{2+}$.

2 Zero Ca$^{2+}$ solutions were actually nominally Ca$^{2+}$-free. EGTA was not included in the modified external medium to buffer a possible contaminating concentration of Ca$^{2+}$. In two cells, zero Ca$^{2+}$ caused a positive shift of the activation and inactivation curves. The values for $V'$ for these cells were not included in the calculation of the shift.

† These shifts, relative to the control values, are significant $p<0.0001$. Significance was determined using analysis of variance.

‡ These shifts were not significant.

¶ This shift was significant ($p<0.0001$). Significance was determined using a paired "t"-test.
TABLE 6. SHIFTS OF THE ACTIVATION AND INACTIVATION CURVES OF $I_k(f)$ CAUSED BY ALTERING THE EXTERNAL DIVALENT CATION CONCENTRATION

<table>
<thead>
<tr>
<th>DIVALENT CATION PRESENT</th>
<th>SHIFT OF THE ACTIVATION CURVE (mV)</th>
<th>SHIFT OF THE INACTIVATION CURVE (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0 μM Cd$^{2+}$</td>
<td>$+8.6 \pm 1.2 \ (4)^\dagger$</td>
<td>N.T.</td>
</tr>
<tr>
<td>200.0 μM Cd$^{2+}$</td>
<td>$+13.1 \pm 1.5 \ (4)$</td>
<td>N.T.</td>
</tr>
<tr>
<td>400.0 μM Cd$^{2+}$</td>
<td>$+16.6 \pm 1.4 \ (4)$</td>
<td>N.T.</td>
</tr>
<tr>
<td>800.0 μM Cd$^{2+}$</td>
<td>$+21.4 \pm 1.9 \ (4)$</td>
<td>N.T.</td>
</tr>
<tr>
<td>1.6 mM Cd$^{2+}$</td>
<td>$+24.3 \pm 1.9 \ (4)$</td>
<td>N.T.</td>
</tr>
<tr>
<td>500.0 μM Cd$^{2+}$</td>
<td>$+24.6 \pm 2.3 \ (5)^\dagger$</td>
<td>$+21.2 \pm 3.9 \ (5)^\dagger$</td>
</tr>
<tr>
<td>2.0 mM Cd$^{2+}$</td>
<td>$+30.1 \pm 1.6 \ (5)^\dagger$</td>
<td>$+33.9 \pm 1.0 \ (5)^\dagger$</td>
</tr>
<tr>
<td>5.0 mM Cd$^{2+}$</td>
<td>$+29.2 \pm 3.7 \ (7)^\dagger$</td>
<td>$+32.8 \pm 3.9 \ (4)^\dagger$</td>
</tr>
<tr>
<td>3 μM Zn$^{2+}$</td>
<td>-2.0 (2)</td>
<td>-1.9 (2)</td>
</tr>
<tr>
<td>31 μM Zn$^{2+}$</td>
<td>$+7.2 \pm 9.5 \ (4)^\ddagger$</td>
<td>$+8.5 \pm 3.3 \ (4)^\dagger$</td>
</tr>
<tr>
<td>62.5 μM Zn$^{2+}$</td>
<td>$+12.8 \pm 2.8 \ (5)^\dagger$</td>
<td>$+14.9 \pm 1.2 \ (4)^\dagger$</td>
</tr>
<tr>
<td>125.0 μM Zn$^{2+}$</td>
<td>$+20.9 \ (2)$</td>
<td>$+19.4 \ (2)$</td>
</tr>
<tr>
<td>250.0 μM Zn$^{2+}$</td>
<td>$+27.5 \ (1)$</td>
<td>$+26.1 \ (1)$</td>
</tr>
<tr>
<td>500.0 μM Zn$^{2+}$</td>
<td>$+26.1 \pm 4.3 \ (4)^\dagger$</td>
<td>$+26.0 \pm 2.5 \ (4)^\dagger$</td>
</tr>
<tr>
<td>0 mM Ca$^{2+}$ (2)</td>
<td>$-11.7 \pm 2.7 \ (4)^\dagger$</td>
<td>$-19.5 \pm 5.2 \ (4)^\dagger$</td>
</tr>
<tr>
<td>5 mM Ca$^{2+}$</td>
<td>$+2.5 \ (2)$</td>
<td>$+7.0 \pm 3.3 \ (4)^\dagger$</td>
</tr>
<tr>
<td>10 mM Ca$^{2+}$</td>
<td>$+8.4 \ (2)$</td>
<td>$+12.3 \pm 3.3 \ (4)^\dagger$</td>
</tr>
<tr>
<td>20 mM Ca$^{2+}$</td>
<td>$+12.4 \pm 2.2 \ (3)$</td>
<td>$+18.2 \pm 3.6 \ (4)^\dagger$</td>
</tr>
<tr>
<td>10 mM Mg$^{2+}$</td>
<td>$-0.8 \pm 4.7 \ (6)^\ddagger$</td>
<td>$+3.0 \pm 1.8 \ (6)^\ddagger$</td>
</tr>
<tr>
<td>40 mM Mg$^{2+}$</td>
<td>$+12.7 \pm 3.9 \ (4)^\dagger$</td>
<td>$+17.2 \pm 3.8 \ (4)^\dagger$</td>
</tr>
</tbody>
</table>
DISCUSSION

Divalent Cations Exert A Charge Screening Effect On The Channel Conducting $I_K(f)$

Changes in the intracellular or extracellular concentration of divalent cations cause the potential-sensitive parameters of voltage-dependent ion channels, such as the activation and inactivation curves, to shift along the voltage axis. Increased divalent cation concentration has also been shown to slow the activation kinetics of voltage-gated ion channels. There are two hypotheses to account for the effects of divalent cations on the gating of voltage-dependent channels. The first suggests that divalent cations neutralized fixed negative charges on the membrane of the cell in a non-specific manner and thus alter the electric field surrounding the voltage sensor (Frankenhaeuser & Hodgkin, 1957; Gilbert & Ehrenstein, 1969; McLaughlin et al., 1971; D'Arrigo, 1978; Hahin & Campbell, 1983). The second hypothesis invokes the specific binding of divalent cations, either to a negatively charged component of the channel protein or to a negatively charged site on or electrically close to the channel (Hille, 1968; Begenisich & Lynch, 1974; Hille et al., 1975; Gilly & Armstrong, 1982 a&b; Mayer & Sugiyama, 1988). Specific binding of divalent cations does not preclude the contribution of non-specific effects as well and surface charge neutralization as a result of both has been proposed to account for the action of divalent cations on sodium channels (Cukierman & Krueger, 1990; Schild et al., 1991). Binding and screening are different ways of neutralizing surface charge but will exert the same effect on the electric field across the membrane (Gilbert & Ehrenstein, 1984).

The charge screening actions of the divalent cations examined here were observed as a reduction in the peak amplitude of $I_K(f)$ resulting from the right-ward shift of both the activation and inactivation curves. In the present study, divalent cation substitution was employed to distinguish between these two mechanisms. The prediction central to non-specific screening of fixed negative surface charges is that cations with the same valence should be equally effective (Hille, 1984). The effects of divalent cations on $I_K(f)$ violate this
prediction. The results reported here suggest that divalent cations likely exert their effects through specific binding either to a site on the channel protein or to a site electrically close to the channel and its voltage sensor.

The divalent cations tested varied widely in their ability to shift the potential-sensitive parameters of $I_K(f)$ along the voltage axis and ranked in the following order:

$$Zn^{2+} > Cd^{2+} >> Ca^{2+} > Mg^{2+}.$$

The fact that the transition metal ions, $Zn^{2+}$ and $Cd^{2+}$, were far more potent than the alkaline earth metal ions, $Ca^{2+}$ and $Mg^{2+}$ is consistent with the rank order reported for the effect of these ions on the sodium channels of both the lobster giant axon and the node of Ranvier of frog myelinated nerve (Blaustein & Goldman, 1968; Hille et al., 1975).

The relationships between the shift of the half-activation potential and the divalent cation concentration, $[X^{2+}]$, were well fitted by the Michaelis-Menton equation,

$$S = S_{max}/(1 + K_M/[X^{2+}]),$$

assuming a single binding reaction. The $K_M$'s for the half-maximal shift of the activation curve varied from 92 $\mu$M for $Zn^{2+}$ to 221 $\mu$M for $Cd^{2+}$ and 3.4 mM for $Ca^{2+}$. The rightward shift ($S$) of the activation curve was saturable but at different concentrations of each species of divalent cation in keeping with the differing $K_M$ values. The positive shifts of $V'$ appeared to saturate at 250 $\mu$M $Zn^{2+}$, 2 mM $Cd^{2+}$, and 20 mM $Ca^{2+}$. The effect of $Mg^{2+}$ was substantially less potent than the other divalents examined and at 40 mM, the highest concentration of $Mg^{2+}$ tested, it is unlikely the action of $Mg^{2+}$ on $I_K(f)$ had saturated.

Given the low potency of $Mg^{2+}$ in shifting the activation and inactivation curves observed, it would be prudent to examine the effect of $Mg^{2+}$ without $Ca^{2+}$ present. Removing $Ca^{2+}$ from the external media resulted in approximately a 12 mV and 19 mV leftward shift of the activation and inactivation curves, respectively. Examining the magnitudes of the $Mg^{2+}$-induced rightward shifts of the activation and inactivation curves in zero $Ca^{2+}$ would be a more sensitive test of the actions of $Mg^{2+}$. 
The results reported here suggest quite strongly that the divalent cations are interacting specifically with a saturable binding site. However, this study did not elucidate whether each of the divalent cations tested was interacting at the same site or whether different binding sites were involved depending on the species of cation. Although 2 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ were present in both the control solutions and the test solutions, other than the solutions in which [Ca$^{2+}$]$_o$ was changed, the effect of each divalent cation on $I_K(f)$ was essentially examined in isolation. It would be of interest to determine whether or not the effects of Zn$^{2+}$ and Cd$^{2+}$ or Zn$^{2+}$ and Ca$^{2+}$ or Ca$^{2+}$ and Mg$^{2+}$, for example, were additive. The effects of changing pH against varied divalent cation concentrations should also shed some light on the nature of the binding site.

Experiments with the frog Node of Ranvier, in which the concentrations of two divalents were varied simultaneously (Mozhayeva & Naumov, 1972c) or the [Ca$^{2+}$]$_o$ and pH$_o$ were varied simultaneously (Hille et al., 1975), suggest that competition exists among divalent cations and between divalent cations and protons for the binding sites present on or near both the potassium channel (Mozhayeva & Naumov, 1972c) and the sodium channel (Hille et al., 1975). If the actions of divalent cations on $I_K(f)$ were additive at saturating concentrations one could postulate that each divalent cation was reacting at a unique saturable binding site, as suggested by Gilly and Armstrong (1982b) based on their observations of the additive effects of Hg$^{2+}$ and Zn$^{2+}$ on the K$^+$ channel of the squid giant axon. If the effects of the divalents were not additive at saturating concentrations this would suggest that the cations were either binding at the same saturable site or that binding of one divalent to its site precluded, by an allosteric effect, binding of the second divalent to its site.

**Divalent Cations Stabilize The Closed Conformation Of The Channel Conducting $I_K(f)$**

The kinetic parameters, $t_{h\,\text{act}}$ and $t_{h\,\text{inact}}$ measured for $I_K(f)$ were shifted along the voltage axis to a greater degree than the activation and inactivation curves. For example, 500 $\mu$M Zn$^{2+}$ evoked a +60 mV shift of both $t_{h\,\text{act}}$ and $t_{h\,\text{inact}}$ compared to a +26 mV shift
of the activation and inactivation curves. The rightward shift of the activation kinetics for $I_K(f)$ in the presence of low concentrations of Cd$^{2+}$ and Zn$^{2+}$ and high concentrations of Ca$^{2+}$ and Mg$^{2+}$ suggest that divalent cations may stabilize the closed conformation of the channel conducting $I_K(f)$, perhaps by binding to a negatively charged component of the gating apparatus in a manner similar to that proposed by Gilly and Armstrong (1982 a & b) for the action of Zn$^{2+}$ on the activation of both Na$^+$ and K$^+$ channels in squid axon.

**Speculation On The Possible Characteristics Of The Binding Site**

Divalent cations could be exerting their action on $I_K(f)$ by binding to a site either on the channel protein itself or near enough to the channel to alter the membrane electric field sensed by the voltage sensor. The results provided in this study suggest the presence of at least one divalent cation specific binding site. The transition metal ions, Cd$^{2+}$ and Zn$^{2+}$, exhibited a much higher affinity for the site than did the alkaline earth metal ions, Ca$^{2+}$ and Mg$^{2+}$.

Zn$^{2+}$ demonstrates a 2-fold higher binding affinity for lipid than Ca$^{2+}$ (Blaustein, 1967) however, this does not seem to account for the 40-fold greater effect of Zn$^{2+}$ and Cd$^{2+}$ in altering the behaviour of $I_K(f)$. On the other hand, transition metal ions bind to proteins with a much higher affinity than Ca$^{2+}$ (Begenisich & Lynch, 1974). Zn$^{2+}$ and Cd$^{2+}$, have respectively, a 234- and 400-fold higher affinity for imidazole groups, a 148- and 280-fold higher affinity for amino groups and a $6.3 \times 10^6$- and $6.3 \times 10^7$-fold higher affinity for sulfhydryl groups relative to Ca$^{2+}$ (Begenisich & Lynch, 1974). The differences in binding affinity to proteins more than accounts for the different potency of the ions tested and suggests strongly that the binding site, for the transition metal ions at least, is part of the channel protein and not on the lipid surrounding the channel.

Begenisich and Spires (1991) exposed the A-type K$^+$ channel from Shaker Drosophila, which has been molecularly characterized, to histidine- or sulfhydryl-modifying agents but found that these agents did not alter the effects of Zn$^{2+}$ on channel behaviour.
However, the amino specific reagent trinitrobenzenesulfonic acid (TNBS), which converts amino groups with a high pK to trinitrobenzene derivatives, completely abolished the $\text{Zn}^{2+}$ effect. It was postulated that the external divalent cation binding site contains one or more important amino residues such as a lysine or a terminal amine (Begenisich & Spires, 1991).

Cahalan and Pappone (1981) utilized TNBS, applied externally to frog skeletal muscle fibres, to increase the negative surface charge on the membrane and observed that TNBS resulted in a left-ward shift of both the activation and inactivation curves for the $\text{Na}^+$ current. In the Begenisich and Spires (1991) study, it is possible that rather than defining an amino component of the divalent cation binding site, TNBS simply counteracted the charge screening effect of the $\text{Zn}^{2+}$ which could have been interacting at an altogether different site.

Schild and Moczydlowski (1991) exposed cardiac sodium channels to iodoacetamide (IAA), a sulfhydryl-specific alkylating agent and found it modified saxitoxin binding and completely abolished the block by $\text{Zn}^{2+}$. Sulfhydryl groups are often present at high-affinity $\text{Zn}^{2+}$ binding sites to coordinate the ion and Schild and Moczydlowski (1991) postulated that the binding site on the cardiac sodium channel contains at least one or more cysteine (Cys) residues. Examination of the amino acid sequence of several potassium channel clones reveals three highly conserved Cys residues located in the first (S1), second (S2) and sixth (S6) transmembrane spanning regions (Bulter et al., 1989; Chandy et al., 1990; Kamb et al., 1988; Schwarz et al., 1988; Stühmer et al., 1989; Swanson et al., 1990; Tempel et al., 1987; Tempel et al., 1988; Wei et al., 1990). Therefore, it is feasible that Cys residues are involved in the $\text{Zn}^{2+}\backslash\text{Cd}^{2+}$ binding site of $\text{I}_\text{K}(f)$ although the evidence provided by Begenisich & Spires (1991) argues against this possibility.

$\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ were less potent than either $\text{Zn}^{2+}$ or $\text{Cd}^{2+}$ is shifting the voltage-sensitive parameters of $\text{I}_\text{K}(f)$. It is conceivable that more than one binding site exists on the channel protein which display different affinities and selectivity for the divalent cations. The dose-response curves fitted to a single binding site do not preclude this possibility as each
divalent cation was essentially examined in isolation. As discussed, the existence of at least two sites for divalent cation action on the delayed rectifier $K^+$ channel in squid axons has been put forward by Gilly and Armstrong (1982b).

**High $[Zn^{2+}]_o$ And $[Cd^{2+}]_o$ Reduce $G_{max}$**

In most instances the apparent reduction of $G_{max}$ observed in the presence of a given $[X^{2+}]_o$ was accounted for by the right-ward shift of the activation curve. If the right-ward shift of the activation curve was substantial $G_{max}$ may not have been attained since the cells were not depolarized to potentials above +80 mV. However, 500 $\mu$M $Zn^{2+}$ and 5 mM $Cd^{2+}$ caused respectively, a 23% and a 20% reduction in $G_{max}$. In both cases, $G_{max}$ was calculated at a membrane potential less depolarized than +80 mV, the maximum potential to which the membrane was depolarized. These results are analogous to those of Mayer and Sugiyama (1988) where they observed a 5-11% and 16-26% reduction in $G_{max}$ with 10 mM and 20 mM $[Mn^{2+}]_o$, respectively, but not with $[Mn^{2+}]_o < 5$ mM.

There is not a clear explanation for the reduction of $G_{max}$ in the presence of high concentrations of $Zn^{2+}$ and $Cd^{2+}$. However, several possibilities suggest themselves. At the macroscopic current level it is difficult to distinguish unequivocally between possible charge screening effects and the voltage-dependent block of $I_K(f)$ by divalent cations. Studies of the effect of $Zn^{2+}$ and $Cd^{2+}$ on the conductance of the cardiac sodium channel indicate that these ions reduce channel conductance by inducing a voltage-dependent conversion of the channel to a subconductance state (Ravindran et al., 1991; Schild et al., 1991). It is possible that $Zn^{2+}$ and $Cd^{2+}$ are having a similar action on $I_K(f)$, however, single channel analysis or noise analysis of macroscopic currents would be required to study this possibility.

The presence of divalent cations in the double layer could decrease the concentration of permeant ions near the mouth of the channel due to electrostatic interactions which would also reduce conductance (Green & Andersen, 1991). For example, Cooper and Shrier (1989) reported that the conductance of A channels in cultured sensory neurones
varied with the square root of the external $K^+$ concentration. If the local concentration of $K^+$ was reduced due to the presence of divalent cations then it is reasonable to expect that the conductance of the channel could be reduced. The reduction in conductance may be the result of the specific interaction of the divalent cations with a site on or close to the channel as well as, non-specific electrostatic effects created when the extracellular concentration of these cations is increased.

In the calculation of the chord conductance $E_R$ was assumed not to have changed when divalent cation concentration was altered, however, the effects of divalent cations on $E_R$ remain to be tested.

**Is $Ca^{2+}$ A Necessary Co-factor For $I_{K(f)}$?**

Removing $Ca_o^{2+}$ significantly increased ($p < 0.0001$) the slope-factor of the activation curve for $I_{K(f)}$ and decreased the peak amplitude of the current. The slope-factor of the inactivation curve was also increased, however, this increase was not significant ($p > 0.01$). The increase in the slope-factor of the activation curve translates into a decrease in the equivalent gating charge transferred across the membrane during the activation process. The reduction of peak $I_{K(f)}$ caused by zero $Ca^{2+}$ is explained by the left-ward shift of the inactivation curve. Although $Ca^{2+}$ has been identified as an essential co-factor in the gating of Na channels in GH$_3$ clonal pituitary cells (Armstrong and Cota, 1991) and $I_A$-like currents (Begenisich, 1988) it is unclear whether $Ca^{2+}$ fulfils a similar role in the gating of $I_{K(f)}$. The change in the slope-factors of the activation curve, in zero $Ca^{2+}$, suggests that $Ca^{2+}$ may play some role in the movement of the gating charge across the membrane during the activation/inactivation processes.

Lack of $Ca^{2+}$ in the bath solution also resulted in an increase in the leak current in melanotrophs. Armstrong, has provided evidence which suggests that $Ca^{2+}$ ions stabilize the closed conformation of delayed rectifier type $K^+$ channels (Armstrong & Matteson, 1986) and are necessary factors in maintaining the functional integrity of these channels
(Armstrong and López-Barneo, 1987). It is not clear from the present study whether the increase in the leak current was due specifically to a loss in the selectivity of the channel carrying $I_K$, as suggested by Armstrong and López-Barneo (1987), or whether the lack of $\text{Ca}^{2+}$ in the external media simply destabilized the membrane and consequently resulted in an increased leakage conductance.

**The Activation and Inactivation Of $I_K$ Appear To Be Coupled**

Equal shifts of both the activation and inactivation curves of $I_K$ suggests that these processes are coupled. The Debye length, which acts as a guide as to how far into a solution the electrostatic effects of a charge can be felt, is less than 10 Å in standard Ringer solution (Hille, 1984). If activation and inactivation were not coupled it is quite feasible that the gating mechanisms governing these two processes would experience very different local electric fields even if they were only 20 Å apart (Hille, 1984). This difference would be observed in the degree to which activation and inactivation were influenced by the presence of divalent cations. As both the activation and inactivation of $I_K$ were affected equally it is more likely that the mechanisms governing these processes experienced the same local electrical field. Inactivation of $I_K$ would become voltage-dependent if it were strongly coupled to activation which is a highly voltage-sensitive process (Armstrong & Bezanilla, 1977). Activation and inactivation could be coupled due to physical constraints imposed by the conformation of the channel protein. Movement of the activation gating particle might be necessary in order to expose a site needed for inactivation to proceed, as proposed in the "ball and chain" model of inactivation (Armstrong and Bezanilla, 1977; Hoshi et al., 1990; Zagotta et al., 1990).

The results with zero $\text{Ca}^{2+}$, on the other hand, suggest that $\text{Ca}^{2+}$ ions in particular are capable of affecting charge transfer during the gating process of activation without influencing the gating charge movement which occurs during inactivation. Zero $\text{Ca}^{2+}$ significantly increased the slope-factor of activation which translates into a reduction in the
equivalent gating charge transferred during the activation process, whereas, there was no significant effect on the slope-factor of the inactivation curve \((p > 0.01)\). This result suggests that the activation process can be acted on separately from the inactivation process by \(\text{Ca}^{2+}\), however, further investigation into the actions of zero \(\text{Ca}^{2+}\) are required to determine whether the action on the slope-factor for inactivation is truly insignificant. Armstrong and Matteson (1986) have proposed that in addition to binding to a negatively charged portion of the gating apparatus, \(\text{Ca}^{2+}\) and perhaps \(\text{Mg}^{2+}\) normally occupies the closed \(\text{K}^{+}\) channel in squid axon. \(\text{Ca}^{2+}\) may operate in a similar fashion at the channel conducting \(I_K(f)\), movement of this ion out of the channel prior to activation could account for some of the charge movement during the activation processes and could explain the reduction in charge movement observed in zero \(\text{Ca}^{2+}\).

**Physico-Chemical Properties Of The Divalent Cations**

Differences in physico-chemical properties, such as, ionic radius, electronegativity, and hydration energy, among the divalent cations tested may explain in part the disparity in their relative abilities to shift the voltage-dependent parameters of \(I_K(f)\). Most notably, \(\text{Mg}^{2+}\), which was the least effective, has a mean hydration shell of approximately 13 water molecules (Arhem, 1980b) and exhibits a water substitution rate of \(10^5 \text{ s}^{-1}\) (Hille, 1984) which is the slowest among the divalent cations examined. The slow replacement of waters around \(\text{Mg}^{2+}\) may be a factor in the ability of \(\text{Mg}^{2+}\) to approach and bind at the putative divalent cation binding site in a manner similar to that described by Hille (1984) to account for the reduced permeability of small ionic channels to \(\text{Mg}^{2+}\), \(\text{Ni}^{2+}\) and \(\text{Co}^{2+}\), each of which holds onto oxygen ligands longer than other inorganic cations. It would be of interest to examine, in detail, the effects of \(\text{Ni}^{2+}\) and \(\text{Co}^{2+}\) on the behaviour of \(I_K(f)\) to determine whether hydration energy is a determining factor in the interaction of divalent cations with the channel conducting \(I_K(f)\). Differences in electronegativity are unlikely to account for the results presented here as \(\text{Mg}^{2+}\) is less electronegative than \(\text{Zn}^{2+}\) or \(\text{Cd}^{2+}\) but more
electronegative than Ca\(^{2+}\). However, the increased electronegativity of Zn\(^{2+}\) and Cd\(^{2+}\) versus Ca\(^{2+}\) and Mg\(^{2+}\) might account in part, for the greater effect on \(I_K(f)\) caused by transition metal ions. Differences in ionic radii are not consistent with the results reported here (Århem, 1980a & 1980b).

**Summary and Future Directions**

Divalent cations evoke a right-ward shift of the potential-sensitive parameters of \(I_K(f)\) along the voltage axis. The ions are not equally potent in their actions which provides compelling evidence that divalent cations exert their effects through interaction with at least one specific and saturable binding site. The presence of divalent cations slows the activation kinetics of \(I_K(f)\) perhaps by stabilizing the \(I_K(f)\) channel in the closed conformation.

Single channel analysis could be used to determine whether divalent cations exert their effects on \(I_K(f)\) solely by shifting the voltage-dependence of gating or, alternatively, the extent of the contribution by voltage-dependent block, or transformation to one or more subconductance states.

At the macroscopic current level, experiments to determine whether divalent cations are competing for the same binding site on the channel conducting \(I_K(f)\) would help quantitate the number of the binding sites.

Exposing melanotrophs to iodoacetamide or TNBS to determine whether either of these agents prevents Zn\(^{2+}\) or Cd\(^{2+}\) from affecting the behaviour of \(I_K(f)\) might help to determine the amino acid components of the binding site for these transition metal ions on the channel conducting \(I_K(f)\).

Site directed mutagenesis could potentially be used to examine whether specific amino acid residues are important in defining a divalent cation binding site in the known potassium channel clones. However, given that the residues involved in forming the binding site might also be involved in forming the channel pore, this technique might not yield definitive results. Moreover, results using this technique must be analyzed keeping in mind
that single amino acid changes can have global effects on channel configuration and that the structure of potassium channels even within a subclass of K⁺ channels such as those subserving A-like currents are quite diverse.

Concluding Remarks

Divalent cations, particularly Cd²⁺, have been used to block voltage-sensitive Ca²⁺ currents in order to isolate other currents of interest (Kehl, 1989) and as a diagnostic tool to determine whether Ca²⁺ influx through voltage-gated Ca²⁺ channels is involved in the activation of other currents (Douglas & Taraskevich, 1982). The results of this study emphasize that even at the concentrations typically used to block Ca²⁺ currents (e.g., 100-300 μM Cd²⁺) the gating of K⁺ channels can be substantially altered.

Protein phosphorylation has been implicated as a mechanism by which the activity of channels conducting the TOC are modulated (DiFrancesco & Tortora, 1991; Braun et al., 1990). The modulatory action of phosphorylation appears in part to involve an alteration of the surface potential sensed by voltage-gated channels through interactions at the cytoplasmic face of the membrane. Phosphorylation of the delayed rectifier K⁺ channel in squid axon has been observed to shift its voltage-dependent parameters and result in modification of its kinetic and conductive properties suggesting that electrostatic interactions may play an important role in modulating the behaviour of voltage-dependent channels (Perozo et al., 1989; Perozo & Bezanilla, 1990). Rudy et al. (1988) have observed that A-currents expressed in Xenopus oocytes required the presence of a large (6-7 kb) and a small (2-4 kb) mRNA species in order to display normal kinetics and pharmacology. They have suggested that the small mRNA species encodes a second subunit of the A-channel which may modulate its behaviour. It is quite feasible that the channel conducting I_K(f) is also modulated by phosphorylation and that part of this modulation involves electrostatic interactions similar to those observed in this study.
BIBLIOGRAPHY


