Modulation Of Cardiac Inward-Rectifier $K^+$ Current
$I_{K1}$ By Intracellular $K^+$ And Extracellular $K^+$

by Oksana Dyachok

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia, Canada
December 2011

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled “Modulation of Inward-Rectifier $K^+$ Current $I_{K1}$ By Intracellular $K^+$ And Extracellular $K^{++}$” by Oksana Dyachok in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The inwardly-rectifying K⁺ current (I_{K1}) is important for heart cell function because it sets the resting potential, influences cell excitability, and promotes repolarization of the action potential. My objective was to investigate the modulation of I_{K1} by extracellular K⁺ (K⁺₀) and intracellular K⁺ (K⁺ᵢ). I_{K1} was recorded from whole-cell-configured guinea-pig ventricular myocytes that were dialyzed with Na⁺-free EGTA-buffered pipette-filling solution and bathed with Na⁺ or NMDG⁺ solution that contained agents that inhibit non-I_{K1} currents.

Lowering K⁺₀ from standard 5.4 to 2 and 1 mM shifted the reversal potential (E_{rev}) of I_{K1} in accord with calculated K⁺ equilibrium potential (E_K), and altered I_{K1} amplitude in accord with conductance (G_{K1})∝ √K⁺₀. Surprisingly, myocytes bathed with 0-mM K⁺ solution had a small outward I_{K1} at holding potential (V_{hold}) ~85 mV. This I_{K1} was attributed to channel-activation by T-tubular K⁺ (K⁺ₜ) whose concentration is sensitive to the flow of T-tubular I_{K1}. K⁺ₜ in myocytes bathed with 0-mM K⁺ solution was ≈ 3.2 mM at V_{hold} ~85 mV, but ≈ 0.3 mM following large K⁺ₜ-depleting flows of inward I_{K1} at ~160 mV. Results consistent with interplay of I_{K1} and K⁺ₜ were also obtained in experiments on myocytes bathed with 2-, 5.4-, and 15-mM K⁺ solution.

Myocytes were dialyzed with pipette solutions that contained 0-140 mM K⁺ to investigate modulation by K⁺ᵢ. When I_{K1} at V_{hold} was kept small, E_{rev} varied with pipette K⁺ in a near-Nernstian manner (i.e., E_{rev} ≈ E_K); however, when I_{K1} (V_{hold}) was large and inward, E_{rev} was markedly negative to nominal E_K. Findings in experiments that involved shifting V_{hold}, changing K⁺₀, and application of Ba²⁺ and Cs⁺ suggest that the magnitude/direction of I_{K1} strongly affects the concentration of K⁺ in submembrane cytoplasm. Classical G_{K1}-voltage parameters G_{K1,max} and V_{0.5} (but not slope factor) were affected by decreases in K⁺ᵢ: G_{K1,max} declined by ≈ 25% per decade decrease in K⁺ᵢ, and V_{0.5} shifted approximately as 0.5 · E_K-shift. The latter findings are discussed and compared with those of earlier studies on the dependence of inwardly-rectifying K⁺ conductance on K⁺ᵢ.
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<tbody>
<tr>
<td>A</td>
<td>ampere</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>silver</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>barium</td>
</tr>
<tr>
<td>Ba₂⁺</td>
<td>extracellular barium</td>
</tr>
<tr>
<td>C⁻</td>
<td>carboxy-</td>
</tr>
<tr>
<td>ca.</td>
<td>approximately</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>cadmium</td>
</tr>
<tr>
<td>chordG_{Kir}</td>
<td>Kir channel chord conductance</td>
</tr>
<tr>
<td>chordG_{Kir,max}</td>
<td>maximal Kir channel chord conductance</td>
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<tr>
<td>chordG_{K1}</td>
<td>K1 channel chord conductance</td>
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<tr>
<td>chordG_{K1,max}</td>
<td>maximal K1 channel chord conductance</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
</tr>
<tr>
<td>COS-1</td>
<td>cell line derived from kidney cells of the African green monkey</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>cesium</td>
</tr>
<tr>
<td>Cs⁺_{pip}</td>
<td>cesium in pipette filling solution</td>
</tr>
<tr>
<td>Ctl</td>
<td>control</td>
</tr>
<tr>
<td>DNFB</td>
<td>2,4-dinitrofluorobenzene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dV</td>
<td>slope of voltage dependence</td>
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<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>$E_K$</td>
<td>$K^+$ equilibrium potential</td>
</tr>
<tr>
<td>$E_{K,b}$</td>
<td>$K^+$ equilibrium potential calculated using bulk external $K^+$</td>
</tr>
<tr>
<td>$E_{K,pip}$</td>
<td>$K^+$ equilibrium potential calculated using pipette-solution $K^+$</td>
</tr>
<tr>
<td>$E_{K,T}$</td>
<td>$K^+$ equilibrium potential calculated using T-tubular $K^+$</td>
</tr>
<tr>
<td>$E_{rev}$</td>
<td>reversal potential</td>
</tr>
<tr>
<td>$G$</td>
<td>conductance</td>
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<td>$G_{Kir}$</td>
<td>non-cardiac inwardly-rectifying $K^+$ channel conductance</td>
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<td>$G_{K1}$</td>
<td>K1 channel conductance</td>
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<td>$G_{K1,T}$</td>
<td>T-tubular membrane K1 channel conductance</td>
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<tr>
<td>$G_m$</td>
<td>membrane conductance</td>
</tr>
<tr>
<td>$G_o$</td>
<td>specific conductance</td>
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<td>$G-V$</td>
<td>conductance-voltage relationship</td>
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<td>$G_{K1-V}$</td>
<td>K1 conductance-voltage relationship</td>
</tr>
<tr>
<td>Glib</td>
<td>glibenclamide</td>
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guinea-pig ventricular myocyte
human embryonic 293 cell line
N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
Hertz (number of cycles per second)
current
current-voltage relationship
I\textsubscript{K1} current-voltage relationship
L-type Ca\textsuperscript{2+}-channel current
holding current
K\textsubscript{ATP}-dependent-channel current
non-cardiac inwardly-rectifying K\textsuperscript{+} current
classical cardiac inwardly-rectifying K\textsuperscript{+} current
I\textsubscript{K1} across T-tubular membrane
rapidly-activating delayed rectifier K\textsuperscript{+} current
slowly-activating delayed rectifier K\textsuperscript{+} current
sodium current
maximal amplitude of outward current
classical transient outward current
concentration that produces 50% inhibition
inside-out
diffusion to/from T-tubules
<table>
<thead>
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<th>Symbol</th>
<th>Description</th>
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<tr>
<td>( K_{\text{ATP}} )</td>
<td>ATP-dependent ( K^+ ) channel</td>
</tr>
<tr>
<td>( K^+ )</td>
<td>potassium</td>
</tr>
<tr>
<td>KCNJ</td>
<td>family of genes of inwardly-rectifying potassium channels</td>
</tr>
<tr>
<td>( K_D )</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kHz</td>
<td>kilohertz</td>
</tr>
<tr>
<td>( K^+_{i} )</td>
<td>intracellular potassium</td>
</tr>
<tr>
<td>Kir</td>
<td>family of inwardly-rectifying ( K^+ ) channels</td>
</tr>
<tr>
<td>Kir2</td>
<td>subfamily of Kir channels</td>
</tr>
<tr>
<td>Kir2.x</td>
<td>a member of Kir2 subfamily (( x = 1 ) to 7)</td>
</tr>
<tr>
<td>( K^+_{o} )</td>
<td>extracellular potassium</td>
</tr>
<tr>
<td>( K^+_{\text{pip}} )</td>
<td>potassium in pipette-filling solution</td>
</tr>
<tr>
<td>( K_T )</td>
<td>T-tubular potassium</td>
</tr>
<tr>
<td>K1</td>
<td>classical cardiac inwardly-rectifying ( K^+ ) channel</td>
</tr>
<tr>
<td>( \text{La}^{3+} )</td>
<td>lanthanum</td>
</tr>
<tr>
<td>log</td>
<td>logarithm</td>
</tr>
<tr>
<td>LQT</td>
<td>long QT interval</td>
</tr>
<tr>
<td>M</td>
<td>mole per litre</td>
</tr>
<tr>
<td>( \text{Mg}^{2+} )</td>
<td>magnesium</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
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<tr>
<td>mM</td>
<td>millimole per litre</td>
</tr>
<tr>
<td>ms</td>
<td>millisecond</td>
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mV  millivolt
n  number of experiments (myocytes)
n  Hill coefficient
nA  nanoampere
nm  nanometer
nM  nanomole per litre
nS  nanosiemens
N-  amino-
Na+  sodium
NMDG+  N-methyl-D-glucamine
P  probability (significance level in statistical test)
P  pore loop
pA  picoampere
pH  negative logarithm of hydrogen ion concentration
pip sol’n  pipette solution
PIP2  phosphatidylinositol-4,5-bisphosphate
pL  picolitre
PKA  protein kinase A
PKC  protein kinase C
PMA  phorbol 12-myristate 13-acetate
PTK  protein tyrosine kinase
QT  interval between Q and T of the electrocardiogram
QR  interval between Q and R of the electrocardiogram
r  radius
R  steady-state rectification
s  second
S  Siemens
S  slope factor
SEM  standard error of mean
Sim  simulated
SPM  spermine
SQTS  short QT syndrome
slopeG_{Kir}  \( G_{Kir} \) from slope of \( I_{Kir} - V \) relation
slopeG_{Kir,max}  maximal slope\( G_{Kir} \)
slopeG_{K1}  \( G_{K1} \) from slope of \( I_{K1} - V \) relation
slopeG_{K1}(E_K)  slope\( G_{K1} \) at/near \( E_K \)
slopeG_{K1,max}  maximal slope\( G_{K1} \)
TM1  transmembrane domain 1
TM2  transmembrane domain 2
\( \mu M \)  micromole per litre
V  volt; voltage
V_{hold}  holding potential
V_m  membrane potential
V_{peak}  voltage at \( I_{peak} \)
V_{test}  test potential
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$V_{0.5}$</td>
<td>half-voltage of Boltzmann function</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>difference (change)</td>
</tr>
<tr>
<td>$\tau$</td>
<td>time constant</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Ohm</td>
</tr>
<tr>
<td>$M\Omega$</td>
<td>megOhm</td>
</tr>
<tr>
<td>$^\circ C$</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>$\sqrt{}$</td>
<td>square root</td>
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<tr>
<td>$\propto$</td>
<td>direct proportionality</td>
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<tr>
<td>$%$</td>
<td>per cent</td>
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<tr>
<td>$\approx$</td>
<td>approximately</td>
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CHAPTER 1. INTRODUCTION

1.1. BRIEF OVERVIEW

$I_{K1}$ is the abbreviation commonly used for the classical inwardly-rectifying $K^+$ current described in studies on cardiac Purkinje fibres and cardiac muscle strands (Hutter & Noble, 1960; Noble, 1965; Dudel et al., 1967; Rougier et al., 1968; Mascher & Peper, 1969) and, later, in early studies on isolated cardiac cells (Isenberg & Klöckner, 1982; Matsuda & Noma, 1984). Since then, $I_{K1}$ has been found in a wide variety of Purkinje fibre cells, ventricular myocytes, and atrial myocytes (Hume & Uehara, 1985; Tourneur et al., 1987; Giles & Imaizumi, 1988; Cohen et al., 1989; Harvey & Ten Eick, 1989; Clark et al., 2001; Bouchard et al., 2004). $I_{K1}$ is constitutively active, and because it is the dominant current at potentials near the resting potential, it is an important determinant of the resting potential in most cardiac cells (Baumgarten & Fozzard, 1992). $I_{K1}$ flows through “$K1$” channels that are complexes of inwardly-rectifying $K^+$ (Kir) subunits that belong to a subfamily of Kir subunits, Kir2 (Jan & Jan, 1997). In addition to expression in the heart, Kir2 is expressed in the central nervous system, skeletal muscle, vascular smooth muscle, and elsewhere (for reviews, see Stanfield et al., 2002; Park et al., 2008; de Boer et al., 2010a; Hibino et al., 2010). Aside from the generation of current-voltage (I-V) relations that display strong inward-rectification (i.e., much larger inward current than outward current), hallmark properties of $I_{K1}$ in cardiac cells (and of Kir2 channel current ($I_{K_{ir}}$) in noncardiac cells) include reversal potential ($E_{rev}$) that is close to the $K^+$ equilibrium potential ($E_K$), activation that is dependent on extracellular $K^+$, and voltage-dependent block by extracellular $Ba^{2+}$ and $Cs^+$ (Stanfield et al., 2002). These properties are amongst those investigated in the present study.
The material below begins with a brief account of the objectives of this study (Section 1.2). That is followed by a general background on Kir2 channels, K1 channels, and I_{K1} (Section 1.3) that is organized as follows: structure, gating, and rectification of Kir2 channels (1.3.1); composition, distribution, and localization of K1 channels (1.3.2); regulation of K1 channels (1.3.3); role of I_{K1} in cardiac function (1.3.4); and conditions that affect I_{K1} and cardiac function (1.3.5). This is followed by a ‘specific background’ section (1.4) that is more closely related to the experimental findings of the study. The areas covered in this section include modulation of I_{K1} by extracellular and intracellular K^+, accumulation and depletion of extracellular K^+, and current flow and possible changes in the concentration of cytoplasmic K^+.

1.2. OBJECTIVES OF THE STUDY

The first objective of this study on guinea-pig ventricular myocytes was to investigate the effects on I_{K1} of lowering the concentration of extracellular K^+ (K^{+o}) from standard 5.4 mM to concentrations as low as 0 mM. The parameters of interest included the reversal potential (E_{rev}) of the current, its peak outward amplitude, and the maximal slope of the inward limb of the I-V relation of the current. It turned out that I_{K1} did not completely disappear when K^{+o} was lowered to nominally 0 mM. This surprising finding led to investigation of possible accumulation and depletion of K^+ in extracellular restricted-diffusion spaces of myocytes.

A further objective of the study was to investigate the status of I_{K1} in myocytes whose concentration of intracellular K^+ (K^{+i}) had been lowered by dialysis with pipette solutions that contained 0-100 mM K^+ (compared to standard 140 mM). The areas of interest in this investigation included (i) E_{rev} and its dependence on the concentration of
K\(^+\) in the pipette solution, and (ii) the relations between \(E_{\text{rev}} \approx E_K\) and descriptors of \(K1\) channel conductance (\(G_{K1}\)). It turned out that \(E_{\text{rev}}\) was strongly influenced by holding potential and holding current, and this finding led to an investigation of whether changes in flows of \(I_{K1}\) caused changes in the concentration of intracellular K\(^+\).

1.3. GENERAL BACKGROUND

1.3.1 Structure, Gating, And Rectification Of Kir Channels

The basic building block of Kir channels is the Kir subunit. At least sixteen Kir subunit genes have been identified and classified into seven subfamilies, Kir1-7. A number of these genes belong to the Kir2 subfamily, including Kir2.1, Kir2.2, Kir2.3, Kir2.4 (de Boer et al., 2010a; Hibino et al., 2010), and, most recently, Kir2.6 in skeletal muscle (Ryan et al., 2010; Dassau et al., 2011). Four subunits associate in homomeric or heteromeric fashion to form a Kir2 channel. Each subunit has two putative transmembrane domains (TM1 and TM2), a linking pore loop (P), and cytoplasmic amino (N) and carboxy (C) terminal domains (Yang et al., 1995; Raab-Graham & Vandenberg, 1998). The current view of Kir channel architecture, which is based on studies of the structure of mammalian Kir2.1 and Kir3.1 channels, as well as on studies of closely-related bacterial channels (KirBac) (e.g., Kuo et al., 2003; Clarke et al., 2010; Paynter et al., 2010) (for reviews, see Anumonwo & Lopatin, 2010; Hibino et al., 2010; Zhou & Jan, 2010), can be summarized as follows: The four pairs of TM1 (outer helix) and TM2 (inner helix) form the transmembrane pore, and support the four P-helices that span the outer leaflet of the membrane. This scaffolding positions a flexible ion selectivity filter atop the central cavity of the (“upper”) pore of the channel. The P-loops contain the GYG
signature sequence important for selectivity of $K^+$. The bases of the four TM2 helices come together to create a narrow region (bundle crossing) that is situated just above the lower or “cytoplasmic” pore. The walls of the cytoplasmic pore are formed by the N- and C-termini, and residues of these termini are likely involved in the modulation of channel activity by intracellular agents such as PIP$_2$ (Logothetis et al., 2007). The cytoplasmic pore extends the ion conduction pathway, and may serve as a concentrating region for cytoplasmic $K^+$ ions (Robertson et al., 2008). The upper region of the cytoplasmic pore has a narrowing called the G-loop (girdle) that may serve as a diffusion barrier (Pegan et al., 2005). Finally, a slide helix at the cytoplasmic end of TM1 is situated outside the TM1 and TM2 helices, lies parallel to the cytoplasmic side of the membrane, and connects the TM1 domain to the N-terminal in the cytoplasmic pore.

_Gating of Kir2 channels._ Kir2 channel gating occurs at a number of different levels. First, it seems likely that conformational changes in the flexible selectivity filter modulate intra-burst openings and closings observed in single-channel current recordings (for review, see Bichet et al., 2003). Second, Kir2 channels have glycine at a TM2 position analogous to that of the “glycine-hinge” in bacterial Kir channels that exhibit gating. It has been proposed that gating at the glycine-hinge might be involved in activation of Kir channels by PIP$_2$ (Zhang et al., 1999; Xie et al., 2008). Third, Pegan et al. (2005) have suggested that the narrow G-loops are gating elements that may participate in the activation of channels by PIP$_2$. Fourth, recent studies on KirBac channels suggest that conformational changes in the cytoplasmic domain correlate with conductive configurations of the selectivity filter, and that the slide helix and bundle crossing play an important role in the conformational changes and channel activation (Clarke et al., 2010; Paynter et al., 2010). Finally, Kir2 channels can be considered to be “gated” by the
intracellular polyvalent cations whose voltage-dependent channel blocking is the basis of inward rectification.

Inward rectification. Inward rectification of Kir channels is due to block of channels by cytoplasmic polyvalent cations, including Mg\(^{2+}\), putrescine, spermidine, and spermine (Matsuda et al., 1987; Ficker et al., 1994; Lopatin et al., 1994). The block is voltage-dependent, being stronger at more positive voltages. The degree of the voltage-dependent block is different for different types of Kir channels. The relatively high-affinity block of Kir2.1 secures “strong” inward rectification in these channels. For example, Dhamoon et al. (2004) found that Kir2.1 and Kir2.1/Kir2.3 channels underwent “complete” rectification, whereas Kir2.3 channels did not.

Of the polyvalent cations noted above, tetravalent spermine appears to be the most important for inward rectification (Lopatin et al., 2000; Lopatin & Nichols, 2001; Lu, 2004). The channel regions that are critical for spermine action are the negatively-charged regions E224/E229 (in the cytoplasmic pore) and D172 (in the transmembrane pore) (Wible et al., 1994; Yang et al., 1995; Pegan et al., 2005). Mutation of spatially-close E224 and E299 causes a slowing and weakening of block by polyamine (Kubo & Murata, 2001). Likewise, mutation of D172 to neutral D172N weakens rectification in Kir2.1 (Stanfield et al., 1994).

The precise details on block by polyamines remain unclear (Anumonwo & Lopatin, 2010; Clarke et al., 2010). A “deep block” model of rectification (Lopatin et al., 1995; John et al., 2004; Kurata et al., 2006; Kurata et al., 2010) holds that spermine molecules may bind near cytoplasmic-pore region E224/E299 without occluding the pore, and then move towards the selectivity filter and bind between D172 and the GYG sequence of the filter in an occlusive manner. An alternative model, the “shallow block” model, proposes...
that the binding of spermine at E229/E299 is occlusive, and that binding at a location between there and D172 stabilizes the block (Guo & Lu, 2003; Shin & Lu, 2005).

In voltage-clamped cardiac cells, the inward rectification of $I_{K1}$ is manifested in the I-V relation as large inward current at voltages negative to $E_K$, and progressively smaller outward current at voltages progressively more positive than $E_K$ (Noble, 1975). Conversion of I-V data to $G_{K1}$-V data indicates that inward rectification reflects a strong decline in $G_{K1}$ (i.e., increased block) with membrane depolarization (Saigusa & Matsuda, 1988; Cohen et al., 1989; Anumonwo & Lopatin, 2010). Changes in the extracellular concentration of $K^+$ change the value of $E_K (\approx -61 \cdot \log(K^+/K^+_o) \approx 35^\circ C)$ and shift $G_{K1}$-V relations along the voltage axis commensurate with the shift in $E_K$ (Sakmann & Trube, 1984; Saigusa & Matsuda, 1988). The reason for the shift in $G_{K1}$-V with $E_K$ may be that the I-V region in which there is strong inward flow of $K^+$ (which effectively knocks intracellular blocking particles off their channel binding sites), shifts along the voltage axis with $E_K$ (Hille, 2001).

An interesting question that can be asked about the relation between the voltage dependence of inward rectification and $E_K$ is whether the voltage dependence shifts with $E_K$ when $E_K$ is shifted via a change in intracellular $K^+$ rather than extracellular $K^+$. Studies on $I_{K1}$ and its counterparts in non-cardiac cell types have yielded conflicting answers on the matter (e.g., Hagiwara & Yoshii, 1979; Vandenberg, 1987; Saigusa & Matsuda, 1988), and it is an area of interest in the present study.

### 1.3.2. Subunit Composition And Localization Of K1 Channels

Kir2.1 is the Kir2 subunit that is most strongly expressed in mammalian ventricular myocytes (Anumonwo & Lopatin, 2010; Hibino et al., 2010); however, Kir2.2 and/or Kir2.3 are also likely to be expressed, perhaps in a species-dependent manner. For
example, both Kir2.1 and Kir2.3 have been detected in dog ventricular myocytes (Melnyk et al., 2002), guinea-pig ventricular myocytes (Warren et al., 2003), fetal mouse ventricular myocytes (Liu et al., 2010), neonatal rat ventricular myocytes (He et al., 2008), and sheep ventricular myocytes (Dhamoon et al., 2004), whereas Kir2.1 and Kir2.2 have been detected in rat ventricular myocytes (Leonoudakis et al., 2001), mouse ventricular myocytes (Panama et al., 2007), and rabbit ventricular myocytes (Zobel et al., 2003; Allah et al., 2011).

The contributions of subunits to the Kir2 channels that carry $I_{K1}$ in guinea-pig ventricular myocytes remain unclear (Hibino et al., 2010). In contrast to the assertion by Liu et al. (2001) that Kir2.2 is the strongest contributor to $I_{K1}$ in guinea-pig ventricular myocytes, others have concluded that Kir2.1 subunits make the largest contribution (Warren et al., 2003; Dhamoon et al., 2004; Yan et al., 2005; Caballero et al., 2010). A major contribution of Kir2.1 to $I_{K1}$ appears to be the case in other types of ventricular myocytes as well (Nakamura et al., 1998; Melnyk et al., 2002; McLerie & Lopatin, 2003; Dhamoon et al., 2004; Harrell et al., 2007).

As noted earlier, $I_{K1}$ is found in both ventricular and atrial myocytes, but it has a much larger density in ventricular myocytes of most species (Hume & Uehara, 1985; Bouchard et al., 2004; Dhamoon et al., 2004; Beckmann et al., 2008), with the exception of mouse (Grandy et al., 2007). In general, $I_{K1}$ density and/or Kir2.1 expression in right ventricular myocytes is the same as (Yao et al., 1999; Gaborit et al., 2007) or moderately lower than (Warren et al., 2003; Veeraraghavan & Poelzing, 2008) that in left ventricular myocytes; within the left ventricle, there appears to be little variation in $I_{K1}$ and expression of Kir2.1 (Schram et al., 2002; Szabó et al., 2005; Kondo et al., 2006). In a
study on adult guinea-pig left ventricle, there was no sex-related difference in either the expression level of Kir2.1 or the density of $I_{K1}$ (Brouillette et al., 2007).

Localization. There is a substantial body of evidence indicating that $I_{K1}$-carrying channels are enriched in the T-tubules of ventricular myocytes. When guinea-pig and rabbit ventricular myocytes are placed in culture, there is a pronounced daily loss of T-tubular membrane (Lipp et al., 1996; Mitcheson et al., 1996; Banyasz et al., 2008) that results in a disproportionately large decline in whole-cell $I_{K1}$ density (Christê, 1999; Banyasz et al., 2008) (but see Beckmann et al. (2008) and Vaidyanathan et al. (2010)). Immunolocalization and electrophysiological studies on mouse ventricular myocytes have indicated that Kir2.1 channels are predominantly localized in the T-tubules (Clark et al., 2001), and that Kir2.3 channels are exclusively localized to T-tubules (Dyachenko et al., 2009). Furthermore, an immunolocalization study on rat ventricular myocytes indicated that both Kir2.1 and Kir2.2 subunits were localized to T-tubules (Leonoudakis et al., 2001). Using immunocytochemistry and immunohistochemistry techniques, Melnyk et al. (2002) found that Kir2.1 was concentrated in the transverse tubules of canine ventricular myocytes, whereas Kir2.3 localized to cell ends. On the other hand, the results of studies on formamide-detubulated rat ventricular myocytes are consistent with uniform density of $I_{K1}$ in T-tubular and surface membrane (Komukai et al., 2002; Swift et al., 2006) (see also Zobel et al., 2003).

1.3.3. Regulation Of $I_{K1}$

1.3.3.1. Phosphorylation Systems

Since each of the Kir2-subunit contributors to $I_{K1}$ has multiple consensus sites for phosphorylation by protein kinase A (PKA), protein kinase C (PKC), and protein tyrosine kinase (PTK) (NetPhos software (Blom et al., 1999)), it is possible that $I_{K1}$ is directly
regulated by one or more of these kinases. However, information on such regulation is sparse and/or conflicting. Koumi et al. (1995) reported that stimulation of PKA activity caused inhibition of $I_{K1}$ in guinea-pig ventricular myocytes, and Vaidyanathan et al. (2010) found that stimulation of $\beta_1$-adrenoceptors with 1 mM isoproterenol caused a strong inhibition of $I_{K1}$ in cultured adult rat ventricular myocytes. In both of these studies, the inhibition was confined to inward-directed $I_{K1}$. In contrast to the foregoing, Sonoyama et al. (2006) reported that stimulation of PKA had little or no effect on $I_{K1}$ in rat ventricular myocytes. Stimulation of $\beta_3$ adrenergic receptors in an expression system caused upregulation of Kir2.1 and Kir2.2, but not Kir2.3 (Scherer et al., 2007), whereas a PKA-stimulating cocktail had moderate mixed effects on Kir2.1 channel current in Cos-1 cells (Vega et al., 2009).

In regard to regulation of $I_{K1}$ by PKC, a low (1 nM) concentration of PKC-activating phorbol ester PMA had no effect on $I_{K1}$ in guinea-pig ventricular myocytes (Tohse et al., 1990), and a 1-µM concentration had no effect on $I_{K1}$ in neonatal rat ventricular myocytes (Puglisi et al., 2011). On the other hand, a 10-nM concentration strongly inhibited $I_{K1}$ in human atrial myocytes (Sato & Koumi, 1995; Karle et al., 2002). PKC activators had little effect on Kir2.1 channel current, but inhibited Kir2.2 and Kir2.3 channel current in expression systems (Zhu et al., 1999; Karle et al., 2002).

In regard to PTK, studies on $I_{K1}$ in guinea-pig ventricular myocytes have concluded that inhibition of the kinase has no effect on the current (Chiang et al., 2002) or decreases its amplitude (Gao et al., 2004). Zhao et al. (2008) and Zhang et al. (2011) have reported that inhibition of PTK has moderate inhibitory effects on Kir2.1 channel currents in *Xenopus* oocytes and HEK293 cells.
1.3.3.2. Phosphatidylinositol-4,5-Bisphosphate (PIP$_2$)

Membrane-anchored PIP$_2$ is an important activator/sustainer of the activity of almost all types of Kir channels (Hilgemann et al., 2001; Xie et al., 2007). Evidence suggests that PIP$_2$ associates with a number of both positively-charged and neutral residues in the cytoplasmic termini, and thereby affects Kir2 channel activity, perhaps by affecting gating at the glycine-hinge point (Lopes et al., 2002; Xie et al., 2008), the G-loop (Pegan et al., 2005), and/or other putative cytoplasmic gate (Logothetis et al., 2007).

In a recent study, D'Avanzo et al. (2010) showed that purified human Kir2.1 and Kir2.2 incorporated in liposomes were directly activated by PIP$_2$. The activation was highly specific, with little or no activation by other phosphoinositides. The same group has presented evidence indicating that a number of anionic phospholipids may also participate in activation of Kir2.1 and Kir2.2 (Cheng et al., 2011b).

1.3.3.3. Cholesterol

Cholesterol, a major lipid component of the cell, has an inhibitory action on the activity of Kir2 channels (Romanenko et al., 2002). A recent study on Kir2.1 by Rosenhouse-Dantsker et al. (2011) suggests that the regulation occurs via cytoplasmic-domain residues that overlap with residues that affect modulation of the channel by PIP$_2$.

1.3.3.4. Extracellular And Intracellular K$^+$

With the exception of Kir7.1 (Krapivinsky et al., 1998), all Kir channels respond to changes in K$^+_o$ with changes in channel conductance and the dependence of conductance on voltage (Hibino et al., 2010). However, the degree to which changes in K$^+_i$ affect these parameters is unclear. More detailed overviews of the effects of K$^+_o$ and K$^+_i$ on I$_{K1}$ and K1 channel conductance are provided at later points below (see Sections 1.3.5 and 1.4).
1.3.4. Role Of $I_{K1}$ In Cardiac Function

$I_{K1}$ is the dominant membrane current in resting nonpacemaker cells of the heart, and this ensures that the cell resting potential is almost as negative as $E_K$ (Baumgarten et al., 1981; Isenberg & Klöckner, 1982; Giles & Imaizumi, 1988; Baumgarten & Fozzard, 1992). The negative resting potential plays a key role in cardiac function: it facilitates recovery of Na$^+$ and Ca$^{2+}$ channels from inactivation induced by action potential depolarization, promotes exchange of extracellular Na$^+$ for intracellular Ca$^{2+}$, helps secure rapid conduction of electrical impulses, and antagonizes development of spontaneous electrical activity (Katzung, 1975; Noble, 1986; Bers, 2001; Pogwizd et al., 2001).

$I_{K1}$ plays an important role in the excitation of cardiac cells. Excitation occurs when excitatory depolarizing current is sufficiently strong to attain action potential threshold and trigger regenerative depolarization in a cell. Outward $I_{K1}$ opposes/offsets excitatory depolarizing current, and consequently can influence the level of the threshold potential (Tourneur et al., 1987; Delmar et al., 1989; McDonald et al., 1989; Dhamoon & Jalife, 2005). Positive (depolarizing) shifts in threshold can in turn slow conduction of the action potential (Veeraraghavan & Poelzing, 2008).

$I_{K1}$ also plays an important role in determining the configuration of the action potential. This modulation is almost fully confined to the repolarizing phases of the action potential because the magnitude of $I_{K1}$ is very small at plateau potentials where rectification of the current is near-complete (Hume & Uehara, 1985; Giles & Imaizumi, 1988; Dhamoon et al., 2004; Fink et al., 2006). However, time- and voltage-dependent inactivation of inward current (contributed primarily by L-type Ca$^{2+}$ current ($I_{Ca,L}$)) and activation of outward delayed-rectifier K$^+$ current promote termination of the plateau and the onset of repolarization (McDonald & Trautwein, 1978; Hume & Uehara, 1985). This
brings the membrane potential to levels (e.g., < −10 mV) that promote removal of rectification. The rate and extent of this removal increase with negative potential, such that $I_{K1}$ becomes a more and more important driver of repolarization as repolarization progresses (Ibarra et al., 1991; Shimoni et al., 1992; Sung et al., 2006; Bányász et al., 2007). This participation of $I_{K1}$ in repolarization affects cardiac function in that the rate of repolarization is a determinant of action potential duration which, in turn, influences $Ca^{2+}$ influx and contraction (Miake et al., 2003; Bouchard et al., 2004; Fink et al., 2006; Romero et al., 2009).

1.3.5. Conditions That Affect $I_{K1}$ and Electrical Activity

1.3.5.1. Changes In Extracellular $K^+$

Since decreases and increases in extracellular $K^+$ affect $E_K$, $G_{K1}$, and the I-V relation of $I_{K1}$ (see Section 1.4.1 below), they affect electrical activity in the heart. Thus, both hypokalemia and hyperkalemia affect $I_{K1}$ and heart electrical activity.

Hypokalemia refers to plasma $K^+ < 3.0 - 3.6$ mM (Farber et al., 1951; Weaver & Burchell, 1960; Braunwald, 2001). The condition may be acute or chronic, and lower serum $K^+$ to ≤ 2 mM in conditions such as primary aldosteronism, familial and non-familial hypokalemic periodic paralysis, chronic diarrhea, and $K^+$-losing nephritis, as well as secondary to diuretic therapy (Weaver & Burchell, 1960; Cheng et al., 2011a). Hypokalemia increases the amplitude of the U-wave of the ECG (Surawicz & Lepeschkin, 1953), most likely as a consequence of a reduction in $I_{K1}$ (Postema et al., 2009). Hypokalemia also predisposes to generation of afterdepolarizations and promotion of a spectrum of ventricular arrhythmias (Davidson & Surawicz, 1967; Sung et al., 2006). These influences are most likely related to both a lowering of $G_{K1}$ and an overloading of
intracellular Ca\textsuperscript{2+} following low-\textit{K}_{o}^-\text{-mediated inhibition of the Na\textsuperscript{+}-K\textsuperscript{+} pump (Eisner & Lederer, 1979; Sung \textit{et al.}, 2006).

It is not only during hyperkalemia (plasma \textit{K}\textsuperscript{+} > 6 mM) that heart cells may experience elevated extracellular \textit{K}\textsuperscript{+}. For example, \textit{K}\textsuperscript{+} may accumulate in the clefts between cells and/or in the narrow T-tubules of cells (see Section 1.4.2 below) during ischemia (Kléber, 1983; Wilde \textit{et al.}, 1990) or even during strenuous exercise (Sejersted & Sjøgaard, 2000). Independent of its origin, an increase in extracellular \textit{K}\textsuperscript{+} depolarizes the membrane, increases resting inactivation of voltage-dependent Na\textsuperscript{+} channels, and enhances outward \textit{I}_{\text{K}1}. These effects are likely to depress excitability, slow the upstroke of the action potential, and slow conduction (Whalley \textit{et al.}, 1994).

\subsection*{1.3.5.2. Non-\textit{K}_{o}^-\text{-Related Conditions That Affect \textit{I}_{\text{K}1} Density}

\textit{I}_{\text{K}1} density is likely to be decreased in hypertrophic and failing hearts (Beuckelmann \textit{et al.}, 1993; Kääb \textit{et al.}, 1998; Pogwizd \textit{et al.}, 2001; Domenighetti \textit{et al.}, 2007; Fauconnier \textit{et al.}, 2010). It is inhibited by a variety of clinical pharmacological agents (Tamargo \textit{et al.}, 2004; Rodriguez-Menchaca \textit{et al.}, 2008; Ponce-Balbuena \textit{et al.}, 2009; de Boer \textit{et al.}, 2010b; El Gebeily & Fiset, 2010; López-Izquierdo \textit{et al.}, 2011), and is down-regulated in patients with longQT syndrome LQT7 due to loss-of-function mutations of the \textit{KCNJ2} gene that encodes for the Kir2.1 subunit (Andersen-Tawil syndrome) (Plaster \textit{et al.}, 2001; Donaldson \textit{et al.}, 2004; Lu \textit{et al.}, 2006; Rajakulendran \textit{et al.}, 2010; Tristani-Firouzi & Etheridge, 2010). On the cellular level, a decrease in \textit{I}_{\text{K}1} density can lower the resting potential, lengthen the action potential duration, and slow repolarization. Such changes in cell electrical activity can result in a lengthening of the Q-R and QT intervals, and predispose to arrhythmia (McLerie & Lopatin, 2003; Nattel, 2003; Sung \textit{et al.}, 2006).
I$_{K1}$ density may be increased by gain-of-function mutations in Kir2 channel proteins (Priori et al., 2005) and by the action of pharmacological agents (Caballero et al., 2010). An increase in I$_{K1}$ can speed up repolarization and shorten the action potential (e.g., Miake et al., 2003). Increases in I$_{K1}$ density via increased activity of Kir2.1 are implicated in short QT syndrome (SQT) (Priori et al., 2005; El Harchi et al., 2009) and in both chronic and familial atrial fibrillation (Bosch et al., 1999; Li et al., 2004; Xia et al., 2005; Girmatsion et al., 2009). Over-expression of Kir2.1 in a transgenic mouse model provokes episodes of ventricular fibrillation (Noujaim et al., 2007), and I$_{K1}$-mediated shortening of the atrial action potential predisposes towards atrial arrhythmogenesis (Zhang et al., 2005; Kharche et al., 2008).

1.4. SPECIFIC BACKGROUND

1.4.1. Effects Of Raising And Lowering K$^+$o On I$_{K1}$ And G$_{K1}$

Effects on I$_{K1}$. It is well known that raising K$^+$o from ca. 5.4 mM to a higher concentration causes a depolarization of the resting membrane of cardiac muscle cells (Gettes et al., 1962; Fozzard & Lee, 1976; Cordeiro et al., 1998), and that the depolarization is primarily related to a positive shift in the E$_{rev}$ of I$_{K1}$ (McDonald & Trautwein, 1978; Boyett et al., 1980; Isenberg & Klöckner, 1982; Cordeiro et al., 1998). Conversely, lowering K$^+$o from ca. 5.4 mM to, say, 2 or 1 mM causes a marked hyperpolarization of the resting membrane (Gettes et al., 1962; Fozzard & Lee, 1976; Bustamante et al., 1981; Isenberg & Klöckner, 1982; Aronson & Nordin, 1988; Gjini et al., 1996; Akuzawa-Tateyama et al., 1998; Bouchard et al., 2004) that is attributed to a negative shift in the E$_{rev}$ of I$_{K1}$ (Isenberg & Klöckner, 1982; Aronson & Nordin, 1988;
Cordeiro et al., 1998; Spindler et al., 1998; Bouchard et al., 2004). The effects of more drastic lowerings of $K_o^+$ on the resting potential are equivocal. For example, there are studies in which a lowering to nominally 0 mM caused marked depolarization of the resting potential that was attributed to abolition of $I_{K1}$ (Fozzard & Lee, 1976; Isenberg & Klöckner, 1982), and others in which a similar lowering caused marked hyperpolarization (without comment on possible underlying mechanisms) (Fan & Liu, 1996; Akuzawa-Tateyama et al., 1998).

In addition to causing a shift in $E_{rev}$, increasing $K_o^+$ has distinct effects on $I_{K1}$-V relations in mammalian cardiac preparations. These include the following: (i) a shift in the entire relation to more positive potentials (Dudel et al., 1967; McDonald & Trautwein, 1978; Isenberg & Klöckner, 1982; Ishihara & Ehara, 1998), (ii) an increase in the slope of the relation near $E_{rev}$ (Dudel et al., 1967; McDonald & Trautwein, 1978; Trautwein & McDonald, 1978; Isenberg & Klöckner, 1982; Sakmann & Trube, 1984), (iii) an increase in the slope of the linear region of the inward limb of the relation (Harvey & Ten Eick, 1988), and (iv) an increase in the amplitude of outward $I_{K1}$ such that the outward limb of the high-$K_o^+$ I-V relation crosses over that of the control-$K_o^+$ relation (Noble, 1975; Trautwein & McDonald, 1978; Boyett et al., 1980; Isenberg & Klöckner, 1982; Sakmann & Trube, 1984; Dhamoon et al., 2004; Gómez et al., 2009).

In contrast to the wealth of quantitative information available on the effects of raising $K_o^+$ on $I_{K1}$, there is far less available on the effects of lowering $K_o^+$ from ca. 5.4 mM. Isolated observations on ventricular myocytes indicate the following directional effects: (i) a shift in $E_{rev}$ to a more negative potential (Aronson & Nordin, 1988; Harvey & Ten Eick, 1988; Ishihara & Ehara, 1998; Bouchard et al., 2004), (ii) a decrease in the slope of the I-V relation at $E_{rev}$ (Isenberg & Klöckner, 1982; Aronson & Nordin, 1988;
Bouchard et al., 2004), (iii) a decrease in the maximal slope of the inward-current limb of the I-V relation (Harvey & Ten Eick, 1988), (iv) a decrease in the maximal amplitude of outward $I_{K1}$ (Isenberg & Klöckner, 1982; Ishihara & Ehara, 1998; Spindler et al., 1998), and (v) a crossing-over of the outward-current limbs of the low-$K^+_o$ I-V relation and the control-$K^+_o$ I-V relation (Isenberg & Klöckner, 1982; Tseng et al., 1987; Aronson & Nordin, 1988; Shimoni et al., 1992; Spindler et al., 1998; Bouchard et al., 2004).

Effects on $G_{K1}$. Early studies on cardiac Purkinje fibres indicated that the slope of the I-V relation (slope$G_{K1}$) at or near estimated $E_K$ (slope$G_{K1}(E_K)$) increased when $K^+_o$ was raised from ca. 5.4 mM (Hall et al., 1963; Noble, 1965; Dudel et al., 1967). The relation between this $G_{K1}$ parameter and $K^+_o$ was not linear. Indeed, in one of the experiments conducted on sheep Purkinje fibres by Dudel et al. (1967), slope$G_{K1}(E_K)$ doubled in magnitude when $K^+_o$ was raised by a factor of four (from 2.7 to 10.8 mM). In retrospect, this response could be taken as an indication that $G_{K1}$ increases approximately as the square root of $K^+_o$ ($\sqrt{K^+_o}$). Subsequent voltage-clamp experiments on guinea-pig and other ventricular muscle preparations indicated that $G_{K1}$-dominated membrane conductance ($G_m$) increased when $K^+_o$ was increased from 3 to 10 mM (McDonald & Trautwein, 1978; Trautwein & McDonald, 1978), and that $G_m$ was larger at voltages negative to $E_K$ than at $E_K$ itself (Beeler & Reuter, 1977; Trautwein & McDonald, 1978; Boyett et al., 1980). Later, Daut (1982) reported that apparent slope$G_{K1}(E_K)$ in guinea-pig ventricular muscles increased approximately as $\sqrt{K^+_o}$ when $K^+_o$ was increased between 3 and 6 mM. Sakmann & Trube (1984) measured the effects of $K^+_o$ (5.4 - 43 mM) on slope$G_{K1}(E_K)$ in guinea-pig ventricular myocytes, and found that the relation between log slope$G_{K1}(E_K)$ and log $K^+_o$ was well-described by a straight line with a slope of $0.54 \pm 0.04$. 
The findings of Daut (1982) and Sakmann & Trube (1984) in regard to slope$G_{K1}(E_K)$ and increases in $K^+_o$ were in line with earlier determinations of the conductances of strong inward-rectifiers in noncardiac preparations ($G_{Kir}$), especially with those of Hagiwara & Takahashi (1974). These investigators measured slope$G_{Kir}(E_K)$ in starfish egg, and found that its magnitude varied approximately with $\sqrt{K^+_o}$. In addition, they determined that the $\sqrt{K^+_o}$ dependence also applied to two other $G_{Kir}$ parameters, slope$G_{Kir,max}$, and chord$G_{Kir,max}$ (where max refers to maximal, and chord$G_{Kir}$ is $I_{Kir}(V)/(V - E_K)$). The equivalent of one of these parameters, slope$G_{K1,max}$, was measured by Harvey & Ten Eick (1988) in a study on cat ventricular myocytes. They reported that the magnitude of slope$G_{K1,max}$ was approximately dependent on $\sqrt{K^+_o}$.

1.4.2. Effects Of Lowered $K^+_i$

Heart cells likely experience a lowering of $K^+_i$ during hypoxia (Baumgarten et al., 1981), osmotic swelling (Lado et al., 1984), and ischemia (Case, 1971; Kléber, 1984). The lowering can be pronounced, as judged by the 50% reduction in the intracellular $K^+$ activity of canine subendocardial Purkinje fibre cells from one-day old infarcts (Dresdner et al., 1987). Intracellular $K^+$ may also be lowered by chronic hypokalemia. For example, the heart cells of cats subjected to a chronic lowering of plasma $K^+$ to 3.3 mM had an intracellular $K^+$ concentration of 92 mM, compared to 154 mM in heart cells from control cats (Harrison et al., 1972).

Heart cells that have lower than normal intracellular $K^+$ activity have depolarized resting potentials (Baumgarten et al., 1981; Dresdner et al., 1987), but whether the normal close dependencies of resting potential on $E_K$, and of $E_{rev}$ ($I_{K1}$) on $E_K$, remain in force is unclear (Matsuda & Noma, 1984; Dresdner et al., 1987; Baumgarten & Fozzard, 1992; Zhabyeyev et al., 2004). One reason to suspect that the latter dependence is
preserved is that Hagiwara & Yoshii (1979) found that the $E_{\text{rev}}$ of $I_{\text{Kir}}$ corresponded to calculated $E_K$ when the $K^+$ concentration of solution perfusing the interior of starfish eggs was lowered from 270 to 125 mM. These investigators also found that in contrast to the effects of raising $K^+_o$ on the $G_{\text{Kir}}$-V relation (i.e., shift along the voltage axis commensurate with the shift in $E_K$), lowering $K^+_i$ had no apparent effect on the $G_{\text{Kir}}$-V relation. However, the situation in regard to $G_{K1}$ in guinea-pig ventricular myocytes may be quite different because Saigusa & Matsuda (1988) found that a lowering of $K^+_i$ from 150 to 50 mM strongly decreased maximal $G_{K1}$ and shifted the $G_{K1}$-V relation along the voltage axis to the same degree as the shift in $E_K$. Saigusa & Matsuda (1988) recorded an increase in maximal outward $I_{K1}$ following a decrease in $K^+_i$, a result that is the opposite of the response observed in other studies on guinea-pig ventricular myocytes with lowered $K^+_i$ (Matsuda & Noma, 1984; Zhabyeyev et al., 2004).

1.4.3. Accumulation And Depletion Phenomena

1.4.3.1. Accumulation Of Extracellular $K^+$

It has long been known that large flows of outward $K^+$ current can cause accumulation of $K^+$ in restricted-diffusion extracellular spaces of experimental preparations of nerve and muscle tissue (Frankenhaeuser & Hodgkin, 1956; Adrian & Freygang, 1962; Eaton, 1972; Orkand, 1980). Since cardiac cells have a variety of well-developed $K^+$ current systems (Dudel et al., 1967; McDonald & Trautwein, 1978; Cordeiro et al., 1998; Roden et al., 2002; Nerbonne & Kass, 2005), and multicellular cardiac preparations have restricted-diffusion extracellular spaces that likely include both intercellular clefts and T-tubules (Sommer & Johnson, 1968; Sommer, 1982; Forbes & van Neil, 1988), it is not surprising that both non-voltage-clamp (Kline et al., 1980) and voltage-clamp experiments on cardiac Purkinje fibres (McAllister & Noble, 1966;
Baumgarten et al., 1977), as well as voltage-clamp experiments on frog atrial trabeculae (Noble, 1976), frog ventricular strips (Cleemann & Morad, 1979), and mammalian ventricular trabeculae and papillary muscles (McDonald & Trautwein, 1978; Boyett et al., 1980) have yielded electrophysiological evidence indicating that large outward flows of $K^+$ through cardiac $K^+$ channels can cause accumulation of extracellular $K^+$. For example, Baumgarten et al. (1977) measured resting potential immediately after termination of a depolarizing clamp step applied to a cardiac Purkinje fibre and found that the magnitude of the positive shift in the resting potential varied with the magnitude of the preceding flow of outward $K^+$ current.

A consistent finding in early studies on accumulation of external $K^+$ due to outward $K^+$ current in nerve and muscle preparations was that for a given-sized current, the apparent degree of accumulation was inversely related to the concentration of $K^+$ in the bathing solution (e.g., Frankenhaeuser & Hodgkin, 1956; Orkand, 1980). A similar type of inverse dependence on bathing solution $K^+$ was also evident in studies of accumulation in multicellular cardiac preparations (e.g., McAllister & Noble, 1966; Baumgarten & Isenberg, 1977; Baumgarten et al., 1977).

It was widely believed that all, or nearly all, of the outward-current-induced accumulation of extracellular $K^+$ in multicellular cardiac preparations was localized to intercellular clefts whose widths can be as narrow as 20-30 nm (Sommer & Johnson, 1968). Consequently, there was little reason for investigators to believe that the extent of current-induced $K^+$ accumulation in experiments on (cleftless) isolated cardiomyocytes could be anything other than very small or negligible (e.g., Isenberg & Klöckner, 1982; Matsuura et al., 1987; Mitsuiye & Noma, 1987; Shah et al., 1987; Main et al., 1997). However, voltage-clamp results obtained by Yasui et al. (1993) indicated that substantial
accumulation of K\(^{+}\) could occur under certain circumstances. These investigators activated ATP-dependent K\(^{+}\) (K\(_{\text{ATP}}\)) channels in guinea-pig ventricular myocytes by treating the myocytes with nicorandil, a selective opener of this type of K\(^{+}\) channel (Hiraoka & Fan, 1989). They found that step-depolarizations of nicorandil-treated myocytes elicited large outward currents through K\(_{\text{ATP}}\) channels (I\(_{\text{K,ATP}}\)), and that repolarizations to a holding potential (−85 mV) that was near E\(_{\text{K}}\) (K\(^{+}\)_o 5.4 mM) elicited time-dependent inward tail currents. The amplitude of the inward tail current was related to the amplitude and the duration of flow of the outward I\(_{\text{K,ATP}}\) on the preceding depolarization, and the decay of the inward tail current (reflecting clearance of the accumulated external K\(^{+}\)) followed a multiexponential time course with a T\(_{1/2}\) < 100 ms. Based on morphological considerations, and on the apparent absence of accumulation phenomena in their experiments on nicorandil-activated I\(_{\text{K,ATP}}\) in guinea-pig atrial myocytes (known to have a paucity of T-tubules (Sommer, 1982)), Yasui et al. (1993) concluded that I\(_{\text{K,ATP}}\)-induced accumulation of extracellular K\(^{+}\) in ventricular myocytes occurs primarily in the T-tubules. Confirmation of key findings of Yasui et al. (1993) was obtained by Tourneur et al. (1994) who used a non-nicorandil channel-opener to activate K\(_{\text{ATP}}\) channels in guinea-pig ventricular myocytes. Knopp et al. (1999) also obtained a number of results similar to those reported by Yasui et al. (1993). They treated guinea-pig ventricular myocytes with 2,4-dinitrophenol and other metabolic inhibitors that lower intracellular ATP and open K\(_{\text{ATP}}\) channels. They found that large flows of outward I\(_{\text{K,ATP}}\) elicited by depolarizations were followed by inward tail currents on repolarizations to −80 mV, and concluded that the inward tails were related to shifts in E\(_{\text{K}}\) to potentials positive to −80 mV as a result of accumulation of K\(^{+}\) in T-tubules.
A further important study on accumulation of extracellular K\textsuperscript{+} in ventricular myocytes was that conducted by Clark et al. (2001) on murine ventricular myocytes. In contrast to the I_{K,ATP}-related protocol of increasing K\textsuperscript{+} outflow used by Yasui et al. (1993) and Knopp et al. (1999), Clark and colleagues secured large outflow of K\textsuperscript{+} into restricted-diffusion extracellular spaces by activating transient outward K\textsuperscript{+} current (I_{to}). They found that large surges of outward K\textsuperscript{+} current elicited by strong depolarizations from holding potential −80 mV were followed by large Ba\textsuperscript{2+}-sensitive inward tail currents on repolarizations. They attributed the inward tail currents to I_{K1} flowing as a consequence of accumulations of K\textsuperscript{+} that shifted E_{rev} to voltages positive to the holding potential of −80 mV. Clark et al. (2001) also reported that accumulation-related inward tails were not observed in experiments on mouse atrial myocytes (which have a low density of T-tubules) (see also Fiset et al., 1997).

1.4.3.2. Depletion Of Extracellular K\textsuperscript{+}

Given that large outward K\textsuperscript{+} current can lead to accumulation of K\textsuperscript{+} in restricted-diffusion extracellular spaces (see above), it is plausible that large inward K\textsuperscript{+} current can cause depletion of K\textsuperscript{+} from these same spaces. Amongst the first to consider K\textsuperscript{+}-current-induced depletion of extracellular K\textsuperscript{+} were Adrian, Almers, and colleagues (e.g., Adrian & Freygang, 1962; Adrian et al., 1970; Almers, 1972; Barry & Adrian, 1973). These investigators recorded inwardly-rectifying K\textsuperscript{+} current (I_{Kir}) from frog skeletal muscle fibres, and found that hyperpolarizing steps to voltages more negative that E_K elicited large inward I_{Kir} that decayed with time during the steps. They attributed much of the decay of the current to a time-dependent depletion of K\textsuperscript{+} in the T-tubules. Reasons for this view included observations that hyperpolarizing pulses caused the E_{rev} of I_{Kir} to shift to more negative potentials, and that both the degree of the negative shift and the degree
of decay of $I_{KIr}$ were much smaller after elevation of the concentration of $K^+$ in the bathing solution.

Results suggesting that depletion of extracellular $K^+$ can also occur in multicellular cardiac preparations were reported by Maughan et al. (1973), Baumgarten & Isenberg (1977), Baumgarten et al. (1977), and McDonald & Trautwein (1978). They found that hyperpolarizing steps applied from holding potentials near $E_{rev}$ elicited inward-going $I_{K1}$ that decayed during the steps. The decays of the currents were concomitant with negative shifts in $E_{rev}$, and attenuated by increases in external $K^+$.

The findings in the aforementioned studies on multicellular cardiac preparations offer little guidance on whether the depletion of $K^+$ occurred in intercellular clefts and/or in T-tubules. However, there have been a number of studies on isolated myocytes that suggest that strong hyperpolarizing pulses can cause depletion of T-tubular $K^+$. For example, Harvey & Ten Eick (1988) observed that 70-ms hyperpolarizations to $-170$ mV shifted the $E_{rev}$ of $I_{K1}$ in cat ventricular myocytes by $-7.1 \pm 1.4$ mV. They also found that inward $I_{K1}$ elicited by hyperpolarizing pulses decayed markedly during the pulses. They attributed part of the decay phases to declines in T-tubular $K^+$ (and, therefore, declines in $K^+$ inward driving force and $K^+$-dependent $G_{K1}$), and part to an “inactivation” of $I_{K1}$ related to the presence of $Na^+$ in the bathing solution. In their study on human ventricular myocytes, Bailly et al. (1998) reported that inward $I_{K1}$ elicited by 200-ms hyperpolarizations to voltages $\leq -140$ mV underwent substantial decay during the pulses. They attributed the decay of $I_{K1}$ to time-dependent block of K1 channels by external $Na^+$, as well as to depletion of $K^+$ in restricted-diffusion extracellular spaces. They suggested that the depletion likely occurred in the T-tubules, and that the finding of strong
attenuation of decay by elevation of $K^+$ from 4 to 20 mM offered strong support for the depletion hypothesis.

Pásek et al. (2003) developed a computer model that is based on a quantitative description of electrical activity in the guinea-pig ventricular myocyte formulated by Luo & Rudy (1994). One purpose of the model was to separate ionic transport systems in “surface” membrane from those in T-tubular membrane to evaluate responses related to events occurring in the T-tubules. Model cylindrical myocytes of 100-µm length and 10-µm radius had T-tubular morphology that was based on the microscopic analysis of rat ventricular myocytes conducted by Soeller & Cannell (1999). The average radius of the tubules was 127 nm, the average length 10 µm, and the fractional volume 3.12%; the tubular membrane represented 69% of the total membrane area (which is considerably larger than estimated for guinea-pig ventricular myocytes, 52.6% (Amsellem et al., 1995)). One set of simulations performed by Pásek et al. (2003) was of electrical activity in a myocyte bathed with 5.4-mM $K^+$ solution and externally stimulated at 1 Hz. These indicated that the $K^+$ concentration in T-tubules increased by ≈ 4.7% during each action potential, and then gradually returned to bulk concentration during the next several hundred milliseconds. They speculated that such accumulation could enhance T-tubular $G_{K1}$ and thereby have a stabilizing effect on the diastolic resting potential of the myocyte; on the other hand, it could have a proarrhythmic influence due to a diminished difference between diastolic resting potential and threshold potential. Subsequent simulations focused on a guinea-pig ventricular model suggested that the increase in T-tubular $K^+$ was ≈ 4% during each action potential (Pásek et al., 2008).

Pásek et al. (2003) also simulated membrane currents in response to voltage-clamp steps. They found that progressively larger inward $I_{K1}$ elicted by progressively stronger
hyperpolarizing pulses to voltages between −100 and −160 mV induced progressively larger depletions of T-tubular K⁺. At steady-state at −160 mV, for example, the concentration of K⁺ in the tubules was just 2 mM. The time-dependent lowering of T-tubular K⁺ early during a pulse was concomitant with marked time-dependent decay of tubular-membrane inward I_K1. Simulated whole-myocyte inward I_K1 displayed less marked decay than tubular I_K1 due to the contribution of non-decaying inward I_K1 in the surface membrane.

1.4.3.3. Current Flow And Possible Changes In Cytoplasmic K⁺

Frankenhaeuser (1962) may have been the first investigator to have speculated on an effect of K⁺ current flow on intracellular K⁺ in a voltage-clamped preparation. He recorded delayed-rectifier K⁺ current from myelinated nerve fibres, and raised the possibility that flows of outward current might cause depletions of K⁺ at the inner edge of the nodal membrane. Other investigators of nerve electrical activity recognized that influxes of Na⁺ during high-frequency stimulation could shift the Na⁺ equilibrium potential by tens of millivolts, and surmised that it might be due to accumulation of the ion in a submembrane space from which it only slowly diffuses to the bulk axoplasm (Nakajima & Onodera, 1969; Bergman, 1970). More recently, the concept that Na⁺ may accumulate within a layer of cytoplasm just below the sarcolemma (“restricted” or “fuzzy space”) of heart cells (Lederer et al., 1990) has become widely accepted. Electron probe X-ray microanalysis studies on guinea-pig and rabbit ventricular myocytes suggest that the restricted-diffusion subsarcolemmal space for Na⁺ may extend 50 to 200 nm below the sarcolemma (Wendt-Gallitelli et al., 1993; Silverman et al., 2003). Estimates of the volume of the fuzzy space for Na⁺ range from < 1% to 10-14% of cell volume (for review, see Török, 2007). However, these estimates may only apply in relation to transmembrane movements of Na⁺ of a particular order of magnitude. For example,
Carmeliet (1992b) has made the point that large influxes of Na$^+$ likely fill up any fuzzy space and spill over into the bulk cytoplasm, and Despa et al. (2004) have shown that it is possible to establish large Na$^+$ gradients in the bulk cytoplasm of rat ventricular myocytes.

Carmeliet (1992b) expressed the view that microheterogeneity of intracellular ions was more likely the rule rather than the exception. In that regard, Hwang et al. (1992) noted the possibility that outward Cl$^-$ current elicited by holding the membrane at a depolarized level might lead to accumulation of Cl$^-$ in the submembrane cytoplasm of a ventricular myocyte, and thereby engender a Cl$^-$ equilibrium potential ($E_{Cl}$) different than that calculated from external Cl$^-$ and the Cl$^-$ concentration in the pipette solution.

Despite the foregoing studies and concepts, there has been little attention paid to the possibility that K$^+$ current flow could affect the concentration of K$^+$ in the cytoplasm of myocytes. The most likely reason for this is that studies on K$^+$ currents in whole-cell configured myocytes are almost always conducted using pipettes that are filled with solutions that contain ca. 140 mM K$^+$, i.e., this concentration of K$^+$ in the cytoplasm, along with diffusion of K$^+$ to and from the pipette, should be able to buffer gains and losses of cytoplasmic K$^+$ related to “typical flows” of outward- and inward-directed K$^+$ current. However, a more careful examination may be warranted when the myocyte is dialyzed with a solution that contains, say, 10 mM K$^+$. The buffering capacity is now very much smaller, and large K$^+$ current flows are likely to create K$^+$ concentration gradients between submembrane cytoplasmic regions where K$^+$ ions have entered or exited, and cytoplasmic regions near the tip of the pipette. The larger and the longer-lasting the current flow, the greater the expected accumulation or depletion of K$^+$ in submembrane cytoplasmic regions, and, consequently, the greater the expected
discrepancy between measured $E_{rev}$ ($\approx E_K$) and $E_K$ calculated as $-61 \cdot \log(K^{+}_{\text{pip}}/K^{+}_{o})$ where $K^{+}_{\text{pip}}$ is the concentration of $K^{+}$ in the pipette-filling solution. A moderating factor in any scenario of accumulation/depletion of $K^{+}$ is the diffusion of $K^{+}$ into/out of the patch-pipette. For the case of a typical ventricular myocyte under typical whole-cell voltage-clamp conditions, Mathias et al. (1990) have calculated that the time constant of exchange of pipette $K^{+}$ and cytoplasmic $K^{+}$ is close to 100 s.
CHAPTER 2. METHODS

All of the experimental data reported in this study were obtained from experiments on guinea-pig ventricular myocytes that were isolated using an enzymatic method, and investigated using a whole-cell voltage-clamp method. Simulated data on the dependence of $I_{K1}$ on voltage and external $K^+$ were obtained using an equation (see below).

2.1. ISOLATION OF MYOCYTES

Guinea pigs weighing 250 - 350 g were sacrificed in accord with national and local regulations on animal experimentation. The animals were anesthetized using isoflurane in 100% oxygen. Hearts were quickly excised, and attached to the base of an 80-cm Langendorff column. The hearts were then retrogradely perfused with normal Tyrode’s solution (see below) for \(\approx 1\) min. Thereafter, they were sequentially perfused with $Ca^{2+}$-free supplemented-Tyrode’s solution for 7 - 9 min, $Ca^{2+}$-free supplemented-Tyrode’s solution containing collagenase (0.1 - 0.15 mg/ml; Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan) for 10 - 12 min, and a high-$K^+$ storage solution for 6 min. The $Ca^{2+}$-free supplemented-Tyrode’s solution contained (in mM) NaCl 125, KCl 4.6, MgCl\(_2\) 1.16, taurine 20, glucose 20, and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) 5 (pH 7.4 with NaOH). The high-$K^+$ storage solution contained (in mM) KCl 30, KOH 80, KH\(_2\)PO\(_4\) 30, glutamic acid 50, MgSO\(_4\) 3, taurine 20, glucose 10, ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) 0.5, and HEPES 10 (pH 7.4 with KOH). All solutions were oxygenated with 100% O\(_2\) and maintained at 37 °C.
Following the final period of heart perfusion, the ventricles were cut into chunks, and myocytes were dispersed by gentle mechanical agitation. The myocyte suspension was filtered through 220-μm polyethylene mesh, and the filtrate was kept in storage solution at room temperature.

2.2. ELECTROPHYSIOLOGICAL RECORDING AND ANALYSIS

An aliquot of myocyte-containing storage solution was dropped into the 0.3-ml main chamber of a plastic and glass bath (Sarstedt, Inc., Newton, NC, USA) positioned on top of the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). After the myocytes had settled on the glass bottom of the chamber, the chamber was perfused with a bathing solution (see below) at a rate of 2-3 ml/min.

Single myocytes were voltage clamped using the conventional whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Pipettes were pulled from thick-walled borosilicate glass capillaries (H15/10/137, Jencons Scientific, Leighton Buzzard, UK) by using the usual two-step process. Pipettes had an inside diameter of 2 - 3 μm, and a resistance of 2 - 3 MΩ when filled with pipette solution and immersed in normal Tyrode’s solution. After establishing a gigaohm seal between the pipette tip and the myocyte surface, a brief surge of suction was applied to rupture the patch of membrane under the pipette tip. Myocytes were lifted off the bottom of the bath to ensure satisfactory superfusion of all myocyte surfaces.

The voltage clamp amplifier used was an EPC-9 (HEKA Electronics, Mahone Bay, NS, Canada). The reference electrode was an Ag-AgCl wire or a DRIREF-2SH electrode (World Precision Instruments, Inc., Sarasota, FL, USA). Series resistance was 4 - 6 MΩ, and compensated by 60 - 70%. Cell capacitance generally ranged from 80 to 140
Membrane currents were filtered at 3 kHz by the amplifier, and digitized at 12 kHz with Pulse software (HEKA Electronics). Data files were converted from Pulse to Axon (Union City, CA, USA) data format, and analyzed with Axon Clampfit software (version 6).

Myocytes that were dialyzed with 140-mM K\textsuperscript{+} pipette-filling solution (see below) were usually held at −85 mV or at potentials that were near calculated E\textsubscript{K}, whereas myocytes that were dialyzed with low-K\textsuperscript{+} pipette-filling solution (see below), were held at predetermined potentials between −10 and −105 mV. Current-voltage (I-V) relations were obtained by using either voltage-ramp or voltage-step protocols. In the voltage-ramp protocols, myocytes were hyperpolarized to a negative potential (e.g., −120 mV) for 2-200 ms and then depolarized at 0.05-0.1 V/s to a selected more positive potential, or were depolarized to a selected ramp-start potential and then hyperpolarized to a selected more negative potential at 0.05-0.1 V/s. In general, voltage ramps were applied every 15-30 s. In voltage-step protocols, myocytes were pulsed from the holding potential to one or more test potentials for 200-1000 ms every 5-30 s. Current amplitudes measured at the ends of pulses were used to construct quasi-steady-state I-V relations. Conductance data were obtained from measurements of the slopes of the linear regions of the inward limbs of I-V relations, or by other procedures detailed in the Results (Chapter 3). Pipette offsets were nulled prior to patch formation and liquid junction potentials (≈ −11 mV for NMDG\textsuperscript{+}-Tyrode’s solution-K\textsuperscript{+} pipette solution combination) were compensated for (−10 mV) during analysis.
2.3. EXPERIMENTAL SOLUTIONS

2.3.1 Bathing Solutions

Myocytes were bathed with a Na\(^+\) Tyrode’s solution that contained (in mM) NaCl 140, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1, glucose 10, and HEPES 5 (pH 7.4 with NaOH), a Na\(^+\) Tyrode’s solution that contained 0 mM K\(^+\) (KCl omitted), or a Na\(^+\) Tyrode’s solution that contained either higher or lower K\(^+\) than 5.4 mM (higher or lower KCl than 5.4 mM, with suitable adjustment of NaCl to maintain normal osmolarity). Alternatively, myocytes were bathed with a Tyrode’s solution similar to the above except that NaCl was replaced by N-methyl-D-glucamine (NMDG\(^+\)) chloride, and the pH was brought to 7.4 with HCl. Glibenclamide (3-5 µM) was added to all bathing solutions except for those in experiments which involved activation of I\(_{K,ATP}\) (see Chapter 3); other ion channel blockers (Cd\(^{2+}\), E4031, and chromanol 293B) were frequently added as well (see below and Chapter 3). The temperature of the bathing solutions was 35-36°C.

A number of the experiments involved application of strong hyperpolarizing pulses. Since these can trigger irregular inward currents through hyperpolarization-induced electropores, solutions were sometimes supplemented with 0.1-0.2 mM La\(^{3+}\) to minimize their occurrence and magnitude (Akuzawa-Tateyama et al., 1998; Song & Ochi, 2002; Dyachok et al., 2010). These concentrations of La\(^{3+}\) have little effect on I\(_{K1}\) in rabbit ventricular myocytes (Akuzawa-Tateyama et al., 1998).

2.3.2 Pipette-Filling Solutions

The standard 140-mM K\(^+\) pipette-filling solution contained (in mM): KCl 30, K\(^+\) aspartic acid 110, Mg-ATP 5, EGTA 5, and HEPES 5 (pH 7.2 with KOH). Pipette
solution that had a concentration of K\(^+\) that ranged from 100 mM to 0 mM was made by replacing K\(^+\) with Cs\(^+\), or, in some experiments, with NMDG\(^+\).

2.4. CHEMICALS

All chemicals used to prepare bathing solutions and pipette-filling solutions were purchased from Sigma-Aldrich (Oakville, ON, Canada) and TOCRIS bioscience (Ellisville, MO, USA).

2.5. DRUGS

In general, drugs were dissolved in vehicle and stored in stock solutions. Stock solutions were kept in the dark at –20 °C, and drugs were added to experimental solutions just prior to experiments.

Organic channel blockers were used as required in order to minimize currents that might overlap with I\(_{K1}\). These agents included the rapidly-activating delayed-rectifier (Kr) channel blocker E4031, the slowly-activating delayed-rectifier (Ks) channel blocker chromanol 293B, and the ATP-dependent K\(^+\) (K\(_{\text{ATP}}\)) channel blocker glibenclamide. E4031 (Eisai, Tokyo, Japan) was dissolved in distilled water, stored as a 10-mM stock solution, and added to bathing solution. Chromanol 293B (Aventis, Strasbourg, France), cromakalim (Sigma-Aldrich), and glibenclamide (TOCRIS bioscience) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), stored as 10- to 100-mM stock solutions, and added to bathing solution.
Other compounds used in the study included spermine (Sigma-Aldrich), 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich), daidzein (Calbiochem, La Jolla, CA, USA), and 17β-estradiol (Sigma-Aldrich). Spermine was dissolved in distilled water, stored as a 10- or 100-mM stock solution, and added to pipette-filling solution. DNFB, daidzein, and 17β-estradiol were freshly prepared in DMSO just before experiments, and added to bathing solutions. The final concentration of DMSO in external solutions was ≤ 0.1%.

2.6. SIMULATION OF $I_{K1}$-V

$I_{K1}$-V relations were simulated in accord with the following assumptions:

1. $I_{K1}$ is a function of $G_{K1}$ and driving force ($V - E_K$): $I_{K1} = G_{K1}(V - E_K)$.

2. $G_{K1}\text{max}$ is dependent on square root of $K_{o}^+$: $G_{K1}\text{max} = G_o \sqrt{K_{o}^+}$, where $G_o = 43.03 \text{ nS/mM}^{-1/2}$, and $G_{K1}\text{max}$ for 5.4 mM $K_{o}^+$ is 100 nS.

3. Steady-state rectification is described by a Boltzmann function as follows:

$$ R = \frac{1}{1 + \exp \left( \frac{V - V_{0.5}}{dV} \right)}, $$

where $V$ is membrane voltage, $V_{0.5}$ is half-voltage of rectification, and $dV$ is slope of rectification. $V_{0.5}$ and $dV$ for 5.4 mM $K_{o}^+$ are −70 mV and −17 mV, respectively.

4. Steady-state rectification shifts with the reversal potential of $I_{K1}$ ($E_{rev}$). That is accomplished by (i) assuming that $dV$ is constant and independent of $K_{o}^+$, and (ii) expressing $V_{0.5}$ as a linear function of $E_K$: $V_{0.5} = E_K + 15$. 
After combining the foregoing assumptions into a single formula, $I_{K1}$ depends on voltage ($V$) and $K^{+}_o (E_K$ set by $K^{+}_o$ and $K^{+}_i$) as follows:

$$I_{K1} = \frac{G_o \sqrt{K_o (V - E_K)}}{1 + \exp \left( \frac{V - (E_K + 15)}{dV} \right)}.$$

$I_{K1}$-$V$ relations were plotted using data analysis software (OriginPro 7; OriginLab, MA, USA) as function graphs with voltage as an independent variable with increment of 0.5 mV, and current as a dependent variable.

### 2.7. STATISTICS

Results are expressed as means ± SEM, with $n$ indicating the number of experiments. Single comparisons were made using Student’s paired or unpaired $t$ test, and multiple comparisons were made using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Differences were considered significant when $P < 0.05$. 
CHAPTER 3. RESULTS

3.1. FEATURES OF $I_{K1}$ IN GUINEA-PIG VENTRICULAR MYOCYTES

Material presented in this section provides a brief overview of features of $I_{K1}$ in guinea-pig ventricular myocytes. This facilitates the presentation of data at later points in this chapter. The section begins with a figure that shows records of currents elicited by clamp steps to voltages between $-170$ and $+80$ mV, and continues by illustrating that the current is sensitive to inhibition by external $Ba^{2+}$ and $Cs^+$, and that the current is modulated by external $K^+$. The section closes with data that illustrate the stability of the current in these myocytes. All of the experiments reported in this section were performed on myocytes that were dialyzed with standard 140-mM $K^+$ pipette solution.

3.1.1. $K^+$ Currents Elicited By Voltage-Clamp Pulses

Figure 1A shows records of membrane currents obtained in an experiment on a myocyte that was bathed with 5.4-mM $K^+$ $Na^+$ solution that contained 0.2 mM $Cd^{2+}$ to block L-type $Ca^{2+}$ current ($I_{Ca,L}$) (McDonald et al., 1994), partially block voltage-dependent $Na^+$ current ($I_{Na}$) (Visentin et al., 1990), and suppress fast-activating $I_{Kr}$ (Sanguinetti & Jurkiewicz, 1990). The myocyte was held at $-40$ mV, and pulsed to potentials between $-100$ and $+80$ mV for 500 ms every 10 s. The traces indicate that the current was near the zero-current level on the pulse to $-85$ mV, large and inward-directed on the pulse to $-100$ mV, moderately large and outward-directed on the pulse to $-55$ mV, and very small and outward-directed on the pulse to $-10$ mV. The currents were increasingly large and outward-directed on the pulses to potentials between $+10$ and $+80$ mV.
Figure 1. Membrane currents recorded from representative guinea-pig ventricular myocytes. The myocytes were superfused with 5.4-mM K⁺ Na⁺ solution that contained 0.2 mM Cd²⁺, and pulsed from the holding potential to other potentials for 500 ms every 10 s. A,B. Panel A: note the relatively flat shape of currents between −100 and −10 mV (indicative of I_{K1}), and the time dependence of current at more positive potentials (indicative of I_{Ks}). Panel B: the I-V relation obtained from measurements of current amplitudes at the ends of the pulses shown in A. C. Records of inward I_{K1} elicited at potentials down to −170 mV in a different myocyte. The dashed lines on the records indicate the zero-current levels.
mV, and had a very different character than the currents on the pulses to potentials between −100 and −10 mV. The latter were relatively flat (time-independent) after completion of early transients, whereas the outward currents at positive potentials increased with time during the pulses. The dependence of these currents on time (“delayed rectification”), the dependence of their activation on voltage, and the outward tail currents that followed them on the repolarizations to −40 mV, identify them as slowly-activating delayed-rectifying $K^+$ currents, i.e., $I_{Ks}$ (Sanguinetti & Jurkiewicz, 1990; Jones et al., 1998). When end-of-pulse current amplitude is plotted against pulse voltage, it is $I_{Ks}$ amplitude that forms the rightmost ascending limb of the I-V relation in Figure 1B. Aside from the ascending limb contributed by $I_{Ks}$, the relation has a pronounced inwardly-rectifying shape that is contributed almost exclusively by $I_{K1}$ between −10 mV and −100 mV (as in Figure 1B), as well as by $I_{K1}$ at more negative potentials (see Figure 1C). The major reason for the predominance of $I_{K1}$ at negative potentials in guinea-pig ventricular myocytes is that it is a relatively large current; by comparison, the amplitudes of basal $Cl^-$ current ($I_{Cl}$) and background $Na^+$ current are negligible (e.g., Matsuda & Noma, 1984).

### 3.1.2. Effects Of Classical $I_{K1}$ Blockers Ba$^{2+}$ And Cs$^+$

As noted above, in the absence of $I_{Na}$ and $I_{Ca,L}$ the predominant current at potentials negative to −10 mV in guinea-pig ventricular myocytes is $I_{K1}$. One way of showing this is to record currents at such potentials, and then apply $I_{K1}$-blocker Ba$^{2+}$. In an experiment on a representative myocyte, 1 mM Cd$^{2+}$ was used to block $I_{Na}$ and $I_{Ca,L}$, 3 µM E4031 to block $I_{Kr}$, and 30 µM chromanol 293B to inhibit any $I_{Ks}$. The myocyte was held at −85 mV and pulsed for 500 ms to more positive and negative potentials at 0.1 Hz. The families of currents in Figure 2A were elicited by pulses to potentials between −100 and
Figure 2. Effects of 1 mM Ba\(^{2+}\) on membrane currents. The myocyte was bathed with 5.4-mM K\(^{+}\) Na\(^{+}\) solution that contained 1 mM Cd\(^{2+}\), 3 µM E4031, and 30 µM chromanol 293B, and pulsed from -85 mV to other potentials at 0.1 Hz. A,B. Records obtained before (Control) (A) and 5 min after addition of 1 mM Ba\(^{2+}\) (B). The dashed line indicates the zero-current level.
−10 mV before (control) and 5 min after application of 1 mM Ba\(^{2+}\). The records indicate that Ba\(^{2+}\) inhibited almost all of the control current.

Like Ba\(^{2+}\), external Cs\(^{+}\) is a classical blocker of I\(_{K1}\) in cardiac ventricular preparations (Trautwein & McDonald 1978; Harvey & Ten Eick, 1988). To evaluate its action on I\(_{K1}\) in guinea-pig ventricular myocytes, myocytes superfused with 5.4-mM K\(^{+}\)NMDG\(^{+}\) solution were held at −85 mV and pulsed with 500-ms steps at 0.1 Hz for determination of end-of-pulse I-V relations just before and 6-8 min after switching to solution that contained Cs\(^{+}\) (equimolar replacement of NMDG\(^{+}\)). The I-V relations obtained in representative experiments with 2 and 10 mM Cs\(^{+}\) indicate that the inhibition of I\(_{K1}\) was dependent on the concentration of the blocker, and that the degree of inhibition increased with negative test potential (Figure 3A,B). For reasons that will be apparent at a later point in relation to myocytes dialyzed with low-K\(^{+}\) pipette solution, it was of value to determine the IC\(_{50}\) at −60 mV in myocytes dialyzed with standard pipette solution. Earlier findings of interest include the following (i) 5 mM Cs\(^{+}\) had no significant effect on I\(_{K1}\) at −60 mV in guinea-pig (Tourneur et al., 1987) and cat (Harvey & Ten Eick, 1988) myocytes, and (ii) 10 mM Cs\(^{+}\) reduced I\(_{K1}\) at −60 mV by ≈ 55% in bovine papillary muscle (Trautwein & McDonald, 1978). Myocytes were treated with a single concentration of Cs\(^{+}\) ranging from 0.1 to 100 mM, and the amplitude of current at −60 mV during steady-state Cs\(^{+}\) action was expressed as a percentage of pre-Cs\(^{+}\) control amplitude. As indicated by the curve in Figure 3C, the data are well described by the Hill equation with an IC\(_{50}\) of 7.5 ± 0.3 mM and a coefficient of 1.13 ± 0.04.

3.1.3. Modulation By External K\(^{+}\)

A hallmark property of I\(_{K1}\) in cardiac preparations is that it is modulated by external K\(^{+}\) (K\(^{+}\)\(_{o}\)). An increase in K\(^{+}\)\(_{o}\) not only shifts the reversal potential (E\(_{rev}\)) of I\(_{K1}\) to a more
Figure 3. Inhibition of $I_{K1}$ by Cs$^+$. Myocytes superfused with 5.4-mM K$^+$ NMDG$^+$ solution that contained 1 mM Cd$^{2+}$ and 5 µM E4031 were held at −85 mV and pulsed to other potentials for 500 ms at 0.1 Hz before (Ctl) and ≈ 7 min after switching to (NMDG$^+$-substituted) Cs$^+$ solution. A,B. Inhibition in myocytes treated with 2 mM Cs$^+$ (A) and 10 mM Cs$^+$ (B). C. Dependence of inhibition at −60 mV on the concentration of Cs$^+$. Current amplitudes measured at the ends of Cs$^+$ treatments were expressed as percentages of amplitudes before treatments (Ctl). The Hill equation fitting the data has an IC$_{50}$ of 7.5 ± 0.3 mM and a coefficient of 1.13 ± 0.04. Number of myocytes in parentheses.
positive potential, it also increases the amplitude of current at given driving forces negative to and positive to $E_{\text{rev}}$ (e.g., Dudel et al., 1967; Trautwein & McDonald, 1978). A decrease in $K^+_{o}$ has the opposite effects of an increase (e.g., Isenberg & Klöckner, 1982), and full removal of $K^+_{o}$ more or less completely shuts off the current (e.g., Matsuda & Noma, 1984; Backx & Marban, 1993).

The foregoing features of modulation of $I_{K1}$ by $K^+_{o}$ were observed in the guinea-pig ventricular myocytes investigated in the present study, and two examples of modulation are illustrated in Figure 4. In the first of these, the I-V relation obtained after raising $K^+_{o}$ to 17.1 mM from 5.4 mM had an $E_{\text{rev}}$ that is shifted to a more positive potential, as well as an increased inward and outward current (Figure 4A). The shift in $E_{\text{rev}}$ was +30 mV in the example experiment, and +32 mV in an additional one (not shown). Thus, the shift in $E_{\text{rev}}$ was close to that expected for a purely-$K^+$ current (which, based on calculations using the Nernst equation, is $-61 \log (5.4/17.1)$, or +30.5 mV).

The second example of modulation of $I_{K1}$ by $K^+_{o}$ is provided by the effect of removing external $K^+$ in a representative experiment. The myocyte was held at −85 mV and pulsed to −50 mV for measurement of the amplitude of outward $I_{K1}$ at that potential before, during, and after replacement of 5.4-mM K$^+$ bathing solution by 0-mM K$^+$ solution. As indicated by the time course of $I_{K1}$ amplitude in Figure 4B, the admission of 0-mM K$^+$ solution rapidly reduced the amplitude of the current from $\approx 580$ pA to $\approx 20$ pA, and re-admission of 5.4-mM K$^+$ solution restored it to its original level.

3.1.4. Stability Of $I_{K1}$

The full recovery of the current in the experiment with 0-mM K$^+$ bathing solution (Figure 4B) provides an example of the stability of $I_{K1}$ in the myocytes investigated in the present study. An examination of the stability was conducted by measuring the amplitude
Figure 4. Modulation of $I_{K1}$ by external $K^+$. The myocytes were bathed with NMDG$^+$ solution that contained 1 mM $Cd^{2+}$, 3 µM E 4031, and 30 µM chromanol 293B. A. Effects of increasing external $K^+$ ($K_o^+$) from 5.4 to 17.1 mM. The myocyte was held at $-85$ mV for the 5.4-mM determination, and then at $-55$ mV for the 17.1-mM determination $\approx 5$ min later. Data points indicate the end-of-pulse amplitudes of currents elicited by 500-ms pulses applied at 0.1 Hz. B. Shut-down of $I_{K1}$ ($-50$ mV) upon replacement of 5.4-mM $K^+$ solution by 0-mM $K^+$ solution.
of outward $I_{K_1}$ at $-50$ mV in control experiments that lasted for at least 30 min. The myocytes were bathed with 5.4-mM $K^+$ NMDG$^+$ solution that contained 1 mM Cd$^{2+}$ and 3 µM E4031, held at $-85$ mV, and pulsed to $-50$ mV. The result obtained in one such experiment is shown in the time plot of Figure 5A, and the overall result from twenty-two experiments conducted over a period of several months is shown in Figure 5B. In the latter experiments, the amplitude of the current measured 30 min after patch-breakthrough was $97 \pm 2\%$ of the amplitude measured 1 min after patch breakthrough.

The data in Figure 5 notwithstanding, there were experiments in which there were marked rundowns in $I_{K_1}$. This behaviour was generally evident during the first five minutes after patch-breakthrough, and these experiments were terminated.

### 3.2. MODULATION OF $I_{K_1}$ BY LOWERING EXTERNAL $K^+$

An objective of the present study was to quantify the effects of lowering $K^+_o$ from 5.4 to 2, 1, and 0 mM on parameters that characterize $I_{K_1}$-V relations in myocytes dialyzed with 140-mM $K^+$ pipette solution. These parameters included $E_{rev}$, maximal slope of the inward limb of the relation ($\text{slope}_{G_{K_1}}^\text{max}$), maximal amplitude of outward $I_{K_1}$, and voltage of maximal (peak) outward $I_{K_1}$. The results of experiments related to this objective are detailed in Section 3.2.1 below. It turned out that the configurations of currents elicited by hyperpolarizing pulses applied to myocytes bathed with 0-mM $K^+$ NMDG$^+$ solution were both novel and unusual. It also turned out that steady-state $I_{K_1}$ was distinctly outward over a wide voltage range when myocytes were superfused with 0-mM $K^+$ NMDG$^+$ solution. As a first step in the investigation of the latter findings, it was important to evaluate the degree to which they were reversible. This evaluation is described in Section 3.2.2 below.
Figure 5. Stability of outward $I_{K1}$ elicited at $-50$ mV. The myocytes were bathed with 5.4-mM $K^+$ NMDG$^+$ solution that contained 1 mM Cd$^{2+}$ and 3 µM E4031. A. Data from a representative experiment. B. Data from experiments on twenty-two myocytes.
3.2.1. Effects Of Lowering $K^+_{o}$ From Standard 5.4 mM

In these experiments, myocytes were equilibrated with 5.4-mM $K^+$ solution for $\approx 10$ min, and end-of-pulse I-V relations were determined using 500-ms pulses from holding potential –85 mV to other potentials. Thereafter, $K^+_{o}$ was lowered from 5.4 mM to 2, 1, or 0 mM, and the test I-V relation determined 5-10 min later. In general, a further I-V relation was determined after subsequent addition of Ba$^{2+}$ to null $I_{K1}$. The principal external cation was NMDG$^+$ except where otherwise noted.

**Lowering $K^+_{o}$ to 2 mM.** I-V relations obtained from a myocyte sequentially superfused with 5.4-mM $K^+$ solution, 2-mM $K^+$ solution, and 2-mM $K^+$ solution that contained 3 mM Ba$^{2+}$ are shown in Figure 6A. Compared to the I-V relation obtained during superfusion with 5.4-mM $K^+$ solution, the one obtained during superfusion with 2-mM $K^+$ solution was shifted by $\approx –25$ mV, and slope$G_{K1}$max was reduced by 32%. The maximum amplitude of outward $I_{K1}$ was smaller than that in the 5.4-mM $K^+$ relation, and occurred at a voltage that was 25-30 mV negative to that in the 5.4-mM $K^+$ relation.

A summary of the results obtained from seven myocytes is provided in Figure 6B. As expected from the high $K^+$ selectivity of K1 channels, lowering $K^+_{o}$ from 5.4 mM to 2 mM had a marked effect on the $E_{rev}$ of $I_{K1}$. The $E_{rev}$ was $–85.4 \pm 1.3$ mV during superfusion with 5.4-mM $K^+$ solution, and $–111.4 \pm 1.9$ mV during superfusion with 2-mM $K^+$ solution ($P < 0.001$). These $E_{rev}$ values are similar to the $E_K$ values of $–86$ and $–112.6$ mV for 5.4 and 2 mM $K^+_{o}$, respectively, calculated on the basis that the concentration of $K^+$ in the cytoplasm was the same as that in the pipette solution. The negative shift in $E_{rev}$ was accompanied by a negative shift in the voltage at which outward $I_{K1}$ reached maximal amplitude; the latter was $–54.9 \pm 2.0$ mV in 5.4-mM $K^+$ solution, and $–83.3 \pm 2.8$ mV in 2-mM $K^+$ solution ($P < 0.001$). Slope$G_{K1}$max declined to 68.2 $\pm 3.5$ % of that determined during superfusion with 5.4-mM $K^+$ solution ($P < 0.001$),
Figure 6. Effects of lowering $K_0^+$ from 5.4 to 2 mM on steady-state $I_{K1}$ parameters. The myocytes were sequentially bathed with NMDG$^+$ solutions that contained 5.4 mM K$^+$, 2 mM K$^+$, and 2 mM K$^+$ + 3 mM Ba$^{2+}$. I-V relations were determined at the end of each 7-min superfusion by using 500-ms pulses applied at 0.1 Hz from holding potential −85 mV (5.4 mM K$^+$) or −105 mV (2 mM K$^+$, Ba$^{2+}$). A. I-V relations from one of the experiments. B. Summary of data from seven experiments. Values in each experiment were determined after subtraction of Ba$^{2+}$ data from 5.4-mM K$^+$ and 2-mM K$^+$ data. Left: effects of K$^+_o$ on $E_{rev}$ and the voltage at peak outward $I_{K1}$. Right: relative magnitudes of $slope G_{K1}\text{max}$ and peak outward $I_{K1}$. † P < 0.001, paired t test.
a degree of decline close to that predicted by a dependence of slopeGK1max on the square root of external K+ concentration (61%). In the outward current region of the I-V, the maximal amplitude of IK1 during superfusion of 2-mM K+ solution was 63.0 ± 3.1% of that measured in the presence of 5.4-mM K+ solution (P < 0.001).

Lowering K+o to 1 mM. I-V relations obtained from a myocyte sequentially superfused with 5.4-mM K+ solution, 1-mM K+ solution, and 1-mM K+ solution that contained 3 mM Ba2+ are shown in Figure 7A. Compared to the I-V relation determined during the superfusion with 5.4-mM K+ solution, the relation determined during the superfusion with 1-mM K+ solution was shifted by about −45 mV and diminished in amplitude both below and above the zero-current voltage level. The lowering of K+o reduced slopeGK1max by about two-thirds, and reduced the maximal amplitude of the outward current by about the same extent.

A summary of the results obtained from seven myocytes is shown in Figure 7B. Decreasing K+o from 5.4 to 1 mM shifted Erev from −84.1 ± 1.1 mV to −129.1 ± 0.9 mV (P < 0.001). The latter value is close to the calculated Ek value of −130.9 mV. The negative shift in Erev was accompanied by a negative shift in the voltage at which outward IK1 reached its maximal amplitude. The voltages at maximal amplitude were −58.3 ± 1.9 mV (5.4 mM K+o) and −98.3 ± 2.8 mV (1 mM K+o) (P < 0.001).

Lowering K+o to 1 mM decreased slopeGK1max to 42.7 ± 5.7% of that measured in control 5.4-mM K+ solution (P < 0.001) (Figure 7B). Although the extent of this decline was very close to the 43% decline predicted by a dependence of slopeGK1max on the square root of external K+ concentration, it is possible that it was overestimated to some degree because the 1-mM K+o slope may have been steeper over a more negative voltage range than over the one that was actually used (−150 to −140 mV). In line with the
Figure 7. Effects of lowering K\textsubscript{o} from 5.4 to 1 mM on steady-state I\textsubscript{K1} parameters. The myocytes were sequentially bathed with NMDG\textsuperscript{+} solutions that contained 5.4 mM K\textsuperscript{+}, 1 mM K\textsuperscript{+}, and 1 mM K\textsuperscript{+} + 3 mM Ba\textsuperscript{2+}, and I-V relations were determined at the end of each 7-min superfusion using 500-ms pulses applied at 0.1 Hz from holding potential −85 mV (5.4 mM K\textsubscript{o}) or −120 mV (1 mM K\textsubscript{o}, 1 mM K\textsubscript{o} + Ba\textsuperscript{2+}). A. I-V relations from one of the experiments. B. Summary of data from seven experiments. Values in each experiment were determined after subtraction of Ba\textsuperscript{2+} data from 5.4-mM K\textsubscript{o} and 1-mM K\textsubscript{o} data. Left: effect of K\textsubscript{o} on E\textsubscript{rev} and the voltage at peak outward I\textsubscript{K1}. Right: relative magnitudes of slopeG\textsubscript{K1}\textsubscript{max} and peak outward I\textsubscript{K1}. † P < 0.001, paired t test.
decline in slope $G_{K1}$, the maximal amplitude of the outward current was reduced to 37.2 ± 3.7% of its 5.4-mM $K^+$ amplitude ($P < 0.001$) (Figure 7B).

Inter-relationships and dependencies. It was of interest to take the data collected in the experiments on lowering $K^+_o$ to 2 and 1 mM and examine how certain parameters might relate to others, and how certain parameters varied with $K^+_o$. Figure 8 shows plots of $E_{rev}$ versus log $K^+_o$, $V_{peak}$ versus $E_{rev}$, $slope_{G_{K1}}$max versus log $K^+_o$, and maximal amplitude of outward current versus log $K^+_o$. The slope of the straight line describing the dependence of $E_{rev}$ on $K^+_o$ has a near-Nernstian value of 61.3 ± 5.1 mV (Figure 8A), and the slope of the straight line fitted to the relation between $V_{peak}$ and $E_{rev}$ has a value of 0.95 ± 0.06 (Figure 8B). The relation between log $slope_{G_{K1}}$max and log $K^+_o$ is well-fitted by a straight line with slope 0.47 ± 0.07 (Figure 8C), whereas the relation between the log of the maximal amplitude of outward current ($I_{peak}$) and log $K^+_o$ is well-fitted by a straight line with slope 0.54 ± 0.07 (Figure 8D).

Lowering $K^+_o$ to 0 mM. A large number of experiments was conducted on myocytes that were superfused with 5.4-mM $K^+$ NMDG$^+$ solution and then with 0-mM $K^+$ NMDG$^+$ solution. The effects of lowering $K^+_o$ from 5.4 to 0 mM were surprising. In particular, hyperpolarizing pulses from holding potential −85 mV elicited large inward currents that declined in a time-dependent manner such that end-of-pulse levels were typically small and outward in direction. A detailed investigation of these phenomena is presented in Section 3.3 below.

3.2.2. Reversibility Of Effects Of Lowering $K^+_o$ To 0 mM

Prior to starting on a more detailed investigation of membrane currents in myocytes bathed with $K^+$-free solution, it was important to examine whether removal of external $K^+$ has deleterious effects on K1 channels that, for example, could be caused by rapid
Figure 8. Dependencies of $I_{K1}$ parameters on $K^+_o$. Same myocytes as in Figures 6 and 7.

A. $E_{rev}$ versus log $K^+_o$. The best-fit straight line ($r^2 = 0.986$) is drawn in accord with $E_{rev} = A + B \cdot \log K^+_o$ where $A = -130.3 \pm 2.4$ mV and $B$ is $61.3 \pm 5.1$ mV.

B. Voltage at maximum outward current ($V_{peak}$) versus $E_{rev}$. The best-fit straight line follows the equation $V_{peak} = A + B \cdot E_{rev}$ where $A = 23.4 \pm 6.4$ mV and $B = 0.95 \pm 0.06$.

C. Log slope $G_{K1,max}$ versus log $K^+_o$. The best-fit straight line ($r^2 = 0.980$) follows the equation $\log G_{K1,max} = A \cdot (K^+_o)^b$ where $A = 46.3 \pm 4.5$ and slope $b = 0.47 \pm 0.07$.

D. Log $I_{peak}$ (maximal outward current) versus log $K^+_o$. The best-fit straight line follows the equation $\log I_{peak} = A \cdot (K^+_o)^b$ where $A = 40.6 \pm 4.1$ and slope $b = 0.54 \pm 0.07$. 
deterioration of myocyte health in K\textsuperscript{+}-free solutions (see Spindler et al., 1998) or by changes in the properties of K\textsubscript{1} pores (as observed in some voltage-dependent K\textsuperscript{+} channels (Melishchuk et al., 1998; Loboda et al., 2001; Wang et al., 2009)). This examination was performed by measuring the responsiveness of I\textsubscript{K1} to elevations of K\textsuperscript{+}\textsubscript{o} following prolonged exposures of myocytes to K\textsuperscript{+}-free solution.

*Elevation of K\textsuperscript{+}\textsubscript{o} to 0.1 and 1 mM.* Five myocytes were superfused with K\textsuperscript{+}-free solution for \(\approx 15\) min, and then superfused with 0.1-mM K\textsuperscript{+} solution for \(\approx 7\) min. The myocytes were held at \(-85\) mV and pulsed with 500-ms steps to more positive and negative potentials near the ends of the two superfusion periods. The amplitudes of the end-of-pulse currents recorded from one of these myocytes are shown in Figure 9. They indicate that the introduction of the K\textsuperscript{+}-containing solution resulted in perceptible increases in outward current amplitude at voltages between \(-100\) and \(-150\) mV, but had little or no effect on outward current amplitude at voltages between \(-90\) and \(-20\) mV. In the five experiments of this type, the 0.1-mM K\textsuperscript{+} solution increased the amplitude of the current at \(-130\) mV by \(22 \pm 8\) pA, but only increased that at \(-85\) mV by \(4 \pm 3\) pA.

Four myocytes were bathed with K\textsuperscript{+}-free solution for 15 min and then bathed with 1-mM K\textsuperscript{+} solution for 5 min. The myocytes were held at \(-85\) mV and pulsed to \(-140\) mV at 0.05 Hz. The amplitudes of the currents at these two potentials at the end of the K\textsuperscript{+}-free exposures were \(+59 \pm 18\) and \(+73 \pm 23\) pA, respectively, whereas the amplitudes at the end of the reactivation period with 1 mM K\textsuperscript{+} were \(+234 \pm 33\) and \(-489 \pm 59\) pA, respectively. The latter values are similar to those measured in experiments in which K\textsuperscript{+}\textsubscript{o} was lowered from 5.4 to 1 mM (see Figure 7A above).

*Elevation of K\textsuperscript{+}\textsubscript{o} to 5.4 mM.* Myocytes were exposed to K\textsuperscript{+}-free solution for a lengthy period, and tested for response to elevation of K\textsuperscript{+}\textsubscript{o}. Examples of time courses of
Figure 9. Effects of raising $K^+_o$ from 0 mM to 0.1 mM. The myocyte was bathed with $K^+$-free NMDG$^+$ solution for $\approx 15$ min, and then with 0.1-mM $K^+$ NMDG$^+$ solution for $\approx 7$ min. The holding potential was $-85$ mV, and pulses to other potentials were applied for 500 ms at 0.1 Hz just before and at the end of the superfusion with 0.1-mM $K^+$ solution.
current amplitudes monitored at various potentials in myocytes undergoing this protocol are shown in Figure 10. In the first example, currents monitored at −55, −85, and −100 mV responded to admission of 5.4-mM K⁺ solution after ≈ 30 min exposure to K⁺-free solution with large increases in outward and inward current at −55 and −100 mV, respectively, and a small increase in outward current at −85 mV (Figure 10A). These changes, which were fully reversible (Figure 10A), indicate that the test solution caused a robust reactivation of I_{K1}. In the second example, current monitored at −90 mV was small (≈ 50 pA) and outward in direction after a 55-min exposure to K⁺-free solution (Figure 10B). It responded to admission of test 5.4-mM K⁺ solution with a marked inward-directed excursion.

The degree of recovery of I_{K1} was evaluated by performing I-V determinations on myocytes that were superfused with 5.4-mM K⁺ solution for 5–7 min (control), exposed to K⁺-free solution for 32.8 ± 1.6 min (n = 5) or 58.8 ± 5.4 min (n = 5), and then superfused with 5.4-mM K⁺ solution once again. In all cases, the myocytes responded to the addition of K⁺ with a reactivation of I_{K1} such that the test steady-state I-V relation resembled the control steady-state relation. A quantitative measure of the degree of reactivation was obtained by comparing test slopeG_{K1,max} with control slopeG_{K1,max}. The test value was 91.0 ± 4.1% of the control value in the myocytes exposed to K⁺-free solution for ≈ 33 min, and 85.2 ± 10.8% of the control in myocytes exposed for ≈ 59 min. These results, together with those on elevation of K⁺o to 0.1 and 1 mM, suggest that K₁ channel function was not unduly affected by relatively long exposures to K⁺-free solution.
Figure 10. Reactivation of $I_{K1}$ following prolonged superfusions with K$^+$-free solution. 

**A, B.** Myocytes were superfused with 0-mM K$^+$ NMDG$^+$ solution for ≈ 34 min (A) and ≈ 57 min (B), and then superfused with 5.4-mM K$^+$ NMDG$^+$ solution for ≈ 7 min. The time plots indicate the amplitudes of currents monitored at $-55, -85$, and $-100$ mV (A) (see voltage schematic), and at holding potential $-90$ mV (B).
3.3. I_{K1} IN MYOCYTES BATHED WITH K^+-FREE SOLUTION

A wide range of experiments was performed on myocytes bathed with K^+-free solution to evaluate the contributions of a number of ionic pathways to steady-state outward current at negative potentials and to transient inward currents elicited by hyperpolarizations. The initial task was to determine the extent of possible contributions by non-I_{K1} currents. This was accomplished by conducting experiments on (i) myocytes that were bathed with K^+-free solution in which Na^+ was the principal cation, and (ii) myocytes that were dialyzed with Cs^+ pipette solution instead of standard K^+ pipette solution (Section 3.3.1 below). The results of these experiments led to the hypothesis that both the steady-state outward current and the time-dependent (transient) inward current reflect K^+ movement through K1 channels; more specifically, that the currents reflect the effects of accumulation and depletion of T-tubular K^+ (K^+_T) on the flow of I_{K1} across T-tubular membrane (I_{K1,T}). The hypothesis was tested by examining the voltage dependence of the inward transient, the effects of external Ba^{2+}, the dependence of tail current configuration on the preceding hyperpolarization, and the effects of low external Cs^+ (Section 3.3.2 below).

3.3.1. Investigation Of Contributions By Non-I_{K1} Currents

3.3.1.1. Findings On Involvement Of Na^+ Channel Current

There were two reasons for performing experiments aimed at evaluating the possible involvement of Na^+ channel current in the steady-state outward current and time-dependent inward current of myocytes bathed with K^+-free solution. The first was connected with the possibility that a fraction of voltage-dependent Na^+ channels in ventricular myocytes may be constitutively active at hyperpolarized potentials (Zilberter
et al., 1994), and the second with the possibility that withdrawal of external K\(^+\) from myocytes may affect channel selectivity (Wang et al., 2009).

**Lack of involvement in steady-state outward current.** The first series of experiments was conducted to evaluate the possibility that a major part of the steady-state outward current at negative potentials was due to outward movement of K\(^+\) through Na\(^+\) channels. If there were such outward K\(^+\)-carried current, bathing myocytes in K\(^+\)-free Na\(^+\) solution rather than K\(^+\)-free NMDG\(^+\) solution should promote large inward Na\(^+\)-carried current that would more than offset outward K\(^+\)-carried current. Myocytes were bathed with 5.4-mM K\(^+\) Na\(^+\) solution for \(\approx 10\) min, and then bathed with K\(^+\)-free Na\(^+\) solution for an additional 7 min. The myocytes were held at \(-80\) mV and pulsed to potentials between \(-110\) and 0 mV for 300 ms at 0.1 Hz for determination of quasi-steady-state (end-of-pulse) I-V relations near the ends of the two superfusions. The results obtained in one of these experiments are shown in Figure 11A. Neither the 5.4-mM K\(^+\) Na\(^+\) I-V relation nor the K\(^+\)-free Na\(^+\) I-V relation was distinguishable from relations determined in experiments with K\(^+\)-free NMDG\(^+\) solution.

Average quasi-steady-state relations from five experiments with Na\(^+\) solution (Figure 11B) indicate that the effects of removal of external K\(^+\) on myocytes bathed with Na\(^+\) solution were not very different than those observed on myocytes that were bathed with NMDG\(^+\) solution. For example, the quasi-steady-state amplitude of the current at \(-100\) mV was \(+25 \pm 38\) pA in the Na\(^+\) series (Figure 11B) versus \(+51 \pm 22\) pA \((n = 6)\) in a parallel NMDG\(^+\) series.

The protocol used in the second series of experiments with Na\(^+\) solution involved patching myocytes coincident with a switch from 5.4-mM K\(^+\) solution to K\(^+\)-free solution, and monitoring current amplitude at constant potential \(-90\) mV. The amplitudes
Figure 11. I-V relations obtained from myocytes bathed with K\(^+\)-free Na\(^+\) solution. The myocytes were bathed with 5.4-mM K\(^+\) Na\(^+\) solution for \(\approx\) 10 min, and then with 0-mM K\(^+\) Na\(^+\) solution for \(\approx\) 7 min. A. I-V relations from an example experiment. B. Average of I-V relations from five experiments. Note that the current amplitude scale is different than that in A.
were generally small and stable over prolonged periods of time. When measured at 10 min post-patch-breakthrough, they were between −25 and +25 pA in 26 of the 42 myocytes investigated. In the remaining 16 myocytes, the currents were larger and more often outward than inward. The overall amplitude of the current at −90 mV was +16 ± 5 pA (n = 42) (data not depicted).

**Lack of involvement in inward transients.** An additional observation in the experiments with K⁺-free Na⁺ solution was that hyperpolarizing steps to voltages below −90 mV elicited time-dependent inward currents that were similar to those observed in experiments with K⁺-free NMDG⁺ solution. Records of inward transients elicited in a representative myocyte bathed with Na⁺ solution are shown in Figure 12A. Both the amplitude and rate of decay of the transient increased with increasing negative voltage in a manner similar to that observed in experiments with NMDG⁺ solution (see below).

The presence of Na⁺ rather than NMDG⁺ in the bathing solution had little effect on the amplitude of the inward transient. This information was obtained from experiments in which myocytes were bathed in K⁺-free NMDG⁺ solution for 10 min, and then in K⁺-free Na⁺ solution for another 10 min. The myocytes were hyperpolarized from −85 to −130 mV for 300 ms near the end of each superfusion, and the difference in current amplitude at 15 ms and 290 ms into the pulse was taken as an estimate of the amplitude of the transient. In eleven experiments, the amplitude of the transient during superfusion with Na⁺ solution was 99.3 ± 5.6% of that measured during the preceding superfusion with NMDG⁺ solution (Figure 12B).

### 3.3.1.2. Lack Of Major Involvement Of Any Non-K⁺ Current

As explained below, the results of experiments in which K⁺ was replaced with Cs⁺ allow conclusions to be drawn concerning possible contributions by various current types to steady-state outward currents and to hyperpolarization-induced inward transients.
Figure 12. External Na$^+$ and inward transients in experiments with K$^+$-free solution. A. Records obtained in a representative experiment. The upper dashed line indicates the zero-current level. B. Lack of effect of switching from K$^+$-free NMDG$^+$ solution to K$^+$-free Na$^+$ solution on the amplitudes of the inward transients elicited by 300-ms pulses to −130 mV (see panel A and schematic). Number of experiments in parentheses.
Effects of replacement of $K_i^+$ by $Cs^+$. Myocytes were dialyzed with $K^+$-free $Cs^+$ pipette solution to evaluate the effects of replacement of $K_i^+$ by $Cs^+$. In practice, the results obtained in experiments with $Cs^+$ pipette solution were compared with those obtained in experiments with $K^+$ pipette solution. The most obvious difference between them was that hyperpolarizations elicited inward transients in $K^+$-dialyzed myocytes, but not in $Cs^+$-dialyzed myocytes (e.g., Figure 13A,B). A second important difference emerged from analysis of currents elicited by 1000-ms pulses to test potentials between $-110$ and $-10$ mV. As indicated by the data shown in Figure 13C, the average end-of-pulse I-V relation determined in twenty experiments with $Cs^+$ pipette solution was more inward-directed than that determined in fifteen experiments with $K^+$ pipette solution. The difference between the two relations was (i) significant at all voltages between $-110$ and $-30$ mV ($P < 0.001$, Tukey-Kramer multiple comparison test), and (ii) larger at voltages below $-90$ mV than at voltages above it. As a point of reference, the amplitude of the current at $-90$ mV was $-19 \pm 3$ pA ($n = 20$) in myocytes dialyzed with $Cs^+$ pipette solution, and $+59 \pm 8$ pA ($n = 15$) in those dialyzed with $K^+$ solution.

The inward shift caused by replacement of $K_i^+$ by $Cs^+$ appears to rule out the possibility that steady-state outward current was *entirely* due to outward $K^+$ current through nonselective cation channels because intracellular $Cs^+$ ions should have moved as easily as intracellular $K^+$ ions through these channels (Isenberg, 1993; Kiyosue *et al.*, 1993) (see also Section 3.3.2.2 below). Rather, the shift suggests that the steady-state outward current in myocytes dialyzed with $K^+$ solution was primarily due to outward movement of $K^+$ through $K^+$-selective channels. Further, the voltage dependence of the shift indicates that the $K^+$ channels had inwardly-rectifying I-V relations.
Figure 13. Effects of Cs⁺ pipette solution on membrane currents and I-V relations in myocytes bathed with 0-mM K⁺ NMDG⁺ solution. **A**, **B**. Records of membrane currents elicited by hyperpolarizing steps in representative myocytes dialyzed with K⁺ pipette solution (K⁺_pip) (A) and Cs⁺ pipette solution (Cs⁺_pip) (B). Note the absence of inward transients in B. The dashed lines indicate the zero-current levels. **C**. Average I-V relations obtained from myocytes dialyzed with K⁺ solution (n = 15) and myocytes dialyzed with Cs⁺ solution (n = 20). Current amplitudes were measured at the ends of 1000-ms pulses applied at 0.1 Hz to potentials between −110 and −10 mV. † P < 0.001 (ANOVA, Tukey-Kramer multiple comparison test).
Replacement of $K^+_i$ by $Cs^+$ eliminated inward transients. This finding rules out participation of $Ca^{2+}$ channel currents in the transients elicited in $K^+$-dialyzed myocytes. Likewise, it rules out possible contributions by inward currents through non-selective cation channels, and by inward currents through anion channels. In brief, this process of elimination leads to the view that the channel type most likely to be central to the generation of the inward transient is an inwardly-rectifying $K^+$ channel.

3.3.2. Investigation Of Contributions By $I_{K1}$

The results of the experiments with external $Na^+$ solution and internal $Cs^+$ solutions suggested that steady-state outward current at negative potentials was due to outward movement of $K^+$ through inwardly-rectifying $K^+$ channels. It seemed highly likely that these were $K1$ channels rather than inwardly-rectifying $K_{ATP}$ channels because the latter should have been (i) inactive due to the high concentration of ATP in the pipette solution, and (ii) blocked by the glibenclamide (3-5 µM) present in all bathing solutions. To examine the contributions of $I_{K1}$ to steady-state currents, inward transients, and tail currents, experiments were conducted using external $Ba^{2+}$ and external $Cs^+$ to block $K1$ channels.

3.3.2.1. Contribution Of $I_{K1}$ To Holding Current At $-85$ mV

The level of current in myocytes dialyzed with $K^+$ pipette solution, bathed with $K^+$-free NMDG$^+$ solution, and held at $-85$ mV was measured just before and 5 min after the addition of $Ba^{2+}$ (1-3 mM). Current records from representative experiments indicate that $Ba^{2+}$ abolished the outward holding current (Figure 14). In twenty-two experiments, the level of the current before addition of $Ba^{2+}$ was $+53.5 \pm 6.8$ pA, and the level after addition was $-13 \pm 2$ pA ($P \lt 0.001$) (Figure 14). It is noteworthy that the magnitude of the current inhibited by $Ba^{2+}$ in these experiments ($67 \pm 6$ pA) is in good agreement with
Figure 14. Effect of Ba$^{2+}$ on holding current in myocytes bathed with K$^+$-free NMDG$^+$ solution. Myocytes dialyzed with K$^+$ pipette solution and bathed with 0-mM K$^+$ NMDG$^+$ solution for $\approx 10$ min were treated with 1-3 mM Ba$^{2+}$ for 5 min. **A.** Superimposed traces of segments of holding currents ($I_{\text{hold}}$) recorded at the holding potential ($V_{\text{hold}}$) of $-85$ mV in three representative experiments. **B.** Summary of $I_{\text{hold}}$ data obtained from 22 experiments (including the 15 K$^+$ pip experiments of Figure 13). $\dagger$ P $<$ 0.001, paired $t$ test. “Difference” data were obtained by subtracting Ba$^{2+}$ data (column b) from corresponding 0-mM K$^+$ data (column a).
the magnitude of the difference in current amplitude at −90 mV between myocytes dialyzed with K⁺ pipette solution and myocytes dialyzed with Cs⁺ pipette solution (average 79 pA, see above).

In a further series of experiments, myocytes bathed with K⁺-free Na⁺ solution were held at −85 mV and treated for 5 min with 1 mM Ba²⁺. The level of the current at −85 mV was +23 ± 11 pA before the addition of the blocker, and −47 ± 8 pA afterwards (n = 10) (P < 0.001) (data not depicted).

3.3.2.2. Contribution Of I_K1 To End-Of-Pulse Currents

Myocytes superfused with K⁺-free NMDG⁺ solution for 10-15 min were treated with a relatively low concentration of Ba²⁺ for 10 min. I-V relations were determined just before and at the end of the treatment using 500-ms pulses from holding potential −85 mV. The results obtained in two representative experiments are shown in Figure 15. In the first of these, a very low (0.01 mM) concentration of Ba²⁺ markedly reduced the amplitude of the steady-state outward current at all potentials between −140 and −20 mV (Figure 15A). In the second, addition of 0.1 mM Ba²⁺ abolished the inward transient and shifted the end-of-pulse current in the inward direction (Figure 15B).

The main series of experiments was conducted using 1 mM Ba²⁺. I-V relations were determined by applying 1000-ms pulses from holding potential −85 mV to voltages between −110 and −30 mV just before and ≈ 7 min after addition of the blocker. The results obtained from fifteen myocytes were averaged, and these values are shown in Figure 16. Ba²⁺ shifted the end-of-pulse current in the inward direction over the entire voltage range, and these shifts were highly significant (P < 0.001) at all potentials negative to −40 mV. The Ba²⁺-sensitive (difference) current (nominally I_K1) had a strong inwardly-rectifying I-V relationship, with mean current amplitude declining from near 80 pA at −110 mV, to 55 and 20 pA at −70 and −40 mV, respectively.
Figure 15. Effects of low concentrations of Ba\(^{2+}\) on membrane currents in myocytes bathed with 0-mM K\(^+\) NMDG\(^+\) solution. The myocytes were held at −85 mV and pulsed with a series of 300- or 500-ms steps at 0.1 Hz before and 10 min after addition of Ba\(^{2+}\). A. End-of-step current amplitudes measured in an experiment with 0.01 mM Ba\(^{2+}\). B. Records obtained before and after addition of 0.1 mM Ba\(^{2+}\). The dashed line indicates the zero-current level.
Figure 16. Effects of 1 mM Ba\(^{2+}\) on the steady-state I-V relations of myocytes bathed with 0-mM K\(^{+}\) NMDG\(^{+}\) solution. The myocytes were held at \(-85\) mV and pulsed to other potentials for 1000 ms at 0.1 Hz just before (0 mM K\(^{+}\)) and 7 min after addition of Ba\(^{2+}\). The data obtained under these two conditions are significantly different (P < 0.001) at all potentials below \(-40\) mV (ANOVA, Tukey-Kramer multiple comparison test).

“Difference” data were obtained by subtracting Ba\(^{2+}\) data from corresponding 0-mM K\(^{+}\) data. The myocytes (n = 15) are the same as those in Figure 13 (K\(^{+}\)pip).
It was of value to compare steady-state I-V relations in myocytes dialyzed with K\(^+\) pipette solution and treated with Ba\(^{2+}\), with steady-state relations in myocytes dialyzed with Cs\(^+\) pipette solution and treated with Ba\(^{2+}\). The K\(^+\)-pipette data were already in hand (Figure 16), and the Cs\(^+\)-pipette data were obtained from sixteen myocytes that were bathed with K\(^+\)-free NMDG solution and treated with 1 mM Ba\(^{2+}\) for \(\approx 5\) min. As indicated by the I-V plot of Figure 17, there is little difference between the two sets of data. This result suggests that the magnitude of one component of the steady-state I-V relation under these experimental conditions, outward-directed nonselective cation current, was similar in K\(^+\)-dialyzed and Cs\(^+\)-dialyzed myocytes. Thus, it seems highly likely that in the case of non-Ba\(^+\)-treated myocytes, the more inward I-V relation in Cs\(^+\)-dialyzed myocytes than in K\(^+\)-dialyzed ones (see Figure 13 above) was entirely or almost entirely related to absence of outward I\(_{K1}\) in Cs\(^+\)-dialyzed myocytes.

### 3.3.2.3. Contribution Of I\(_{K1}\) To Inward Transients

The absence of inward transients on hyperpolarizations of myocytes dialyzed with Cs\(^+\) solution provides an indirect indication of the involvement of I\(_{K1}\) in the transients. A more direct indication is provided by the finding that application of 0.1 mM Ba\(^{2+}\) abolished the transient (Figure 15). Supporting evidence was obtained in experiments with I\(_{K1}\)-blocker Cs\(^+\). Myocytes bathed with K\(^+\)-free NMDG\(^+\) solution were held at \(-85\) mV and hyperpolarized to \(-120\) mV for 200 ms every 10 s before and during application of 0.3 mM Cs\(^+\). After 3-min application of the blocker, the amplitude of the hyperpolarization-induced transient (difference in current levels at 15 and 190 ms post-pulse-onset) was reduced to 37 ± 4% of its pre-Cs\(^+\) value (n = 10 myocytes) (P < 0.001) (Figure 18). The inhibition was reversible, i.e., 3-min washouts of Cs\(^+\) from four test myocytes produced recoveries of transients to 94 ± 5% of pre-Cs\(^+\) amplitudes.
Figure 17. Steady-state I-V relations obtained from Ba\(^{2+}\)-treated myocytes dialyzed with K\(^+\) and Cs\(^+\) pipette solutions. The myocytes were bathed with K\(^+\)-free NMDG\(^+\) solution and treated with 1 mM Ba\(^{2+}\) for 5-7 min. The K\(^+\)-dialyzed-myocyte data (K\(_{\text{pip}}^{+}\) + Ba\(_{o}^{2+}\); n = 15) (from Figure 16) are not significantly different than the Cs\(^+\)-dialyzed-myocyte data (Cs\(_{\text{pip}}^{+}\) + Ba\(_{o}^{2+}\); n = 16).
Figure 18. Reduction of the amplitude of the inward transient by external Cs\(^+\). Myocytes were superfused with 0-mM K\(^+\) NMDG\(^+\) solution for 10-15 min and then with similar solution that contained 0.3 mM or 5 mM Cs\(^+\) for an additional 3 min. The myocytes were held at \(-85\) mV and pulsed to \(-120\) mV for 200 ms at 0.1 Hz. The amplitudes of the transients were measured as the difference in current levels at 15 and 190 ms post-pulse-onset, and expressed as percentages of pre-Cs\(^+\) (0 mM K\(^+\)) values. † P < 0.001, paired t test.
Additional experiments were conducted using 5 mM Cs\(^+\). This concentration reduced the amplitude of the transient to 4 ± 1% of its pre-Cs\(^+\) amplitude (n = 9 myocytes) (P < 0.001) (Figure 18). In brief, the effects of Cs\(^+\) on the amplitude of the transient were similar to those of Ba\(^{2+}\).

3.3.2.4. Contribution Of I\(_{K1}\) To Tail Currents At −85 mV

*Tails following small-amplitude pulses.* The traces shown in Figure 19 illustrate the configurations of the tail currents that followed small-amplitude voltage steps. These particular tails were recorded in an experiment on a myocyte that was bathed in K\(^+\)-free NMDG\(^+\) solution, held at −85 mV, and pulsed to −110, −100, −90, −80, and −70 mV for 500 ms at 0.1 Hz before and after addition of 0.1 mM Ba\(^{2+}\). The tail currents that followed the hyperpolarizing pulses were outward in relation to the holding current level, and they decayed by > 70% within 100-ms of their onsets (Figure 19A). By contrast, the tail current that followed the depolarizing pulse to −70 mV was markedly inward in relation to the holding current level (Figure 19A). Although not shown in the figure, the tail current that followed the depolarization from −85 to −80 mV was also inward, but smaller in amplitude than the tail that followed the −70-mV pulse. Both outward- and inward-directed tail currents were abolished by addition of 0.1 mM Ba\(^{2+}\) (Figure 19B), leading to the conclusion that they were carried by K1 channels. That being the case, the outward-directed tail currents in question must have been caused by hyperpolarization-induced increases in G\(_{K1}\) and/or hyperpolarization-induced increases in outward driving force on K\(^+\) ions. Since these increases likely declined with time after termination of the hyperpolarizing pulses, they likely account for the time dependencies of the tails. In regard to whether the tail was induced by an increase in G\(_{K1}\) and/or an increase in driving force, the following may be noted: (i) it was difficult to discern whether or not G\(_{K1}\)
Figure 19. Tail currents elicited after small-amplitude hyperpolarizing and depolarizing pulses. The myocyte was bathed with K$^+$-free NMDG$^+$ solution for 15 min, and then with K$^+$-free solution that contained 0.1 mM Ba$^{2+}$ for an additional 7 min. The dashed lines indicate the zero-current level. A. Records obtained at the end of the superfusion with K$^+$-free solution. Note that the tail current after the pulse to $-70$ mV was inward-directed, whereas the tail currents after pulses to more negative potentials were outward-directed. B. Records obtained at the end of the superfusion with 0.1 mM Ba$^{2+}$ solution.
increased during hyperpolarizing pulses; and (ii) by contrast, it is highly likely that hyperpolarization (inward current)-induced depletion of $K^+_{T}$ (see below) resulted in transient increases in post-hyperpolarization outward driving force.

The traces in Figure 19A indicate that with respect to the holding current, the tail current was outward after the pulse to $-90$ mV and inward after the pulse to $-70$ mV. Scrutiny of the configurations of the currents recorded during the pulses to these potentials provides little reason for the change in tail current direction, i.e., the currents almost superimpose on each other, with the exception of oppositely-directed small transients at early times. It may be that the decaying inward tail current after the pulse to $-70$ mV reflects a clearance of $K^+$ accumulated during the pulse, as well as a time-dependent recovery of $I_{K1}$ from the rectification that occurred during the pulse.

*Tails following large-amplitude hyperpolarizations.* Tail currents following hyperpolarizing steps from $-85$ mV to $-90$, $-100$, and $-110$ mV were always similar to the ones shown in Figure 19A, i.e., they were time-dependent and outward with respect to the level of the holding current at $-85$ mV. Tail currents following hyperpolarizing steps to more negative voltages such as $-140$ mV were different; they were always *inward* with respect to the holding current level. An example of this dependence of tail direction on pulse voltage is provided by the traces shown in Figure 20. They were obtained by subtracting records obtained during treatment of a myocyte with 0.1 mM Ba$^{2+}$ from those obtained before treatment. The $I_{K1}$ tail on termination of the hyperpolarizing pulse to $-150$ mV was inward with respect to the steady-state holding current; it was largest just after the termination, and declined to near-zero over the next 500 ms. A plausible explanation for this behaviour is that the depletion of $K^+_{T}$ during the pulse resulted in a
Figure 20. Dependence of $I_{K1}$ tail configuration on the amplitude of the preceding hyperpolarization. The myocyte was bathed with 0-mM $K^+$ NMDG$^+$ solution for 10 min, and then treated with 0.1 mM $Ba^{2+}$ for 5 min. The currents shown are difference currents (pre-$Ba^{2+}$ minus $Ba^{2+}$). The dashed line indicates zero difference current.
lowering of $G_{K1,T}$ and a subsequent dampening effect on tail current amplitude that more than offset the stimulatory effect of increased outward driving force.

3.4. OUTWARD $I_{K1}$ AND ACCUMULATION OF EXTRACELLULAR $K^+$

Findings presented in Section 3.3 suggest that outward $I_{K1}$ can cause accumulation of $K^+$ in restricted-diffusion extracellular spaces of myocytes that are bathed with 0-mM $K^+$ solution. The purpose of experiments described below was to evaluate whether outward $I_{K1}$ can cause accumulation of $K^+$ in myocytes bathed with 5.4-mM $K^+$ solution. The first step in this investigation was to follow earlier findings by others in regard to accumulation elicited by large outward $I_{K1,ATP}$.

3.4.1. Accumulation Mediated By Outward Flow Of $I_{K1,ATP}$

In the seminal study on $K^+$-current-induced accumulation of $K^+$ in restricted extracellular spaces of guinea-pig ventricular myocytes, the prerequisite large flow of outward $K^+$ current that engendered the hallmark inward tail current on repolarization was attained by activating $K_{ATP}$ channels with nicorandil (Yasui et al., 1993). In the present study, we used the $K_{ATP}$-activation approach to evoke accumulation as a prelude to investigation of accumulation mediated by $I_{K1}$. Figure 21A shows results obtained from a myocyte that was treated with creatine kinase inhibitor DNFB (1 mM) to activate $I_{K1,ATP}$ (Lawrence et al., 2002; Ryu et al., 2005). The myocyte was bathed with 5.4-mM $K^+$ $Na^+$ solution, held at $-80$ mV, and pulsed to potentials up to $+20$ mV for 500 ms at 0.1 Hz. The current records obtained after activation of $I_{K1,ATP}$ featured inward tail currents whose amplitudes increased with those of the preceding outward $I_{K1,ATP}$ and whose time courses could be described by monoexponential functions with $\tau$’s that
**Figure 21.** Inward tail currents associated with flows of outward $I_{K,ATP}$ and $I_{K1}$.  

A. Current records obtained from a myocyte that was bathed in 5.4-mM $K^+$ $Na^+$ solution and treated with 1 mM DNFB for $\approx 15$ min to activate $I_{K,ATP}$. Left: records showing outward $I_{K,ATP}$ elicited by depolarizations, and associated inward tail currents elicited by repolarizations (arrow). Right: records indicating marked inhibition of both outward $I_{K,ATP}$ and inward tail current shortly after application of 10 $\mu$M glibenclamide (Glib).

B. Current records obtained in an experiment on a myocyte that was bathed with 5.4-mM $K^+$ NMDG$^+$-blocker solution, held at $-85$ mV, and pulsed to voltages between $-90$ and $-40$ mV for 500 ms at 0.1 Hz. The records were obtained just before (Ctl) and 5 min after addition of 1 mM Ba$^{2+}$. The lower diagonal arrow on the left points to tails associated with the pulses to $-70$, $-60$, and $-50$ mV. The dashed lines on records indicate zero-current levels.
ranged from 77 to 91 ms. Both outward $I_{\text{K,ATP}}$ and inward tail current were strongly diminished by $I_{\text{K,ATP}}$ blocker glibenclamide (10 µM) (Figure 21A, right). Similar glibenclamide-sensitive inward tail currents were recorded on repolarizations to −80 mV in other experiments with DNFB (n = 6) and $K_{\text{ATP}}$-activator cromakalim (50 µM) (n = 5).

In marked contrast to the foregoing, but in agreement with Yasui et al. (1993), inward tail currents were absent in myocytes bathed and dialyzed with $K^+$-free solution and treated with $K_{\text{ATP}}$ channel opener (n = 5) (data not depicted). Thus, these results point to the following relation between $K^+$ outflow on depolarization and inward tail current on repolarization (cf. Yasui et al., 1993): (i) large outward $I_{\text{K,ATP}}$ during a depolarizing pulse causes an accumulation of $K^+$ in narrow restricted-diffusion T-tubules, (ii) this elevation of T-tubular $K^+$ ($K^+_T$) shifts the applicable $E_K$ ($E_K,T$) to a voltage above holding potential −80 mV, and (iii) repolarization to −80 mV elicits inward $K^+$ current that decays in concert with the clearance of accumulated $K^+$ via diffusion to bulk extracellular space and $K^+$-channel passage to the cytoplasm.

### 3.4.2. Accumulation Mediated By Outward Flow Of $I_{\text{K1}}$

To investigate the possibility that an outward flow of $I_{\text{K1}}$ on depolarization can cause an accumulation of $K^+$ that evokes an inward tail current on repolarization, experiments were conducted on myocytes in which $I_{\text{K,ATP}}$ was suppressed by inclusion of 5 mM MgATP in the pipette solution and 5 µM glibenclamide in the bathing solution. Non-$I_{\text{K1}}$ current components were minimized by using NMDG$^+$ bathing solution that contained channel blockers Cd$^{2+}$ (1 mM) and E4031 (3 µM) in addition to glibenclamide (NMDG$^+$-blocker solution). Current records obtained from a representative myocyte bathed with 5.4-mM $K^+$ NMDG$^+$-blocker solution are shown in Figure 21B. The myocyte was held at −85 mV and pulsed to more positive and negative potentials for 500 ms at 0.1
Hz just before (control) and 5 min after addition of $I_{K1}$-blocker $Ba^{2+}$ (1 mM). The control records indicate that the end-of-pulse amplitude of the current varied with the amplitude of the pulse in a manner characteristic of inwardly-rectifying $I_{K1}$. Flows of outward current were followed by inward tail currents, and both of these current components were almost completely abolished by the addition of $Bal^{2+}$. In six experiments of this type, the amplitude of the tail current (obtained by extrapolation of monoexponential tail-fits to zero-time) following pulses to $-50$ mV was $-169 \pm 46$ pA. In a larger group of myocytes ($n = 35$), the ratio of the amplitude of the inward tail current to that of the outward current at $-50$ mV was $-0.19 \pm 0.02$. The time constants of decay of tail currents ranged from 40 to 95 ms.

Effects of $I_{K1}$-blocker $Cs^{+}$ on outward $I_{K1}$ and inward tail current are shown in Figure 22A. The records in the figure were obtained from a myocyte that was held at $-85$ mV and pulsed to $-75$ and $-80$ mV for 500 ms at 0.1 Hz before and during 5-min superfusions with 2- and 10-mM $Cs^{+}$ solutions. The pulses elicited outward $I_{K1}$, and the repolarizations to $-85$ mV elicited inward tail currents that decayed with time constants near 45 ms. The amplitudes of the tails were roughly proportional to those of the preceding outward $I_{K1}$, and $Cs^{+}$ decreased both components in a concentration-dependent manner. Similar results were obtained in five other experiments of this type.

Another approach that was used to establish a linkage between outward $I_{K1}$ and inward tail current was to determine the effects of certain $I_{K1}$-inhibitory organic compounds on the tail current. Amongst these compounds were daidzein, $17\beta$-estradiol, and spermine. Each of these agents decreased the amplitude of outward $I_{K1}$ and concomitantly decreased the amplitude of the inward tail current. $I_{K1}$-inhibitor daidzein ($IC_{50} \approx 62$ µM (Chiang et al., 2002)) was applied at a concentration of 100 µM for $\approx 7$
Figure 22. Inhibitory effects of $I_{K1}$ blockers on outward $I_{K1}$ on depolarization and inward tail current on repolarization. The myocytes were bathed with 5.4-mM K$^+$ NMDG$^+$-blocker solution, held at $-85$ mV, and pulsed to other potentials for 500 ms at 0.1 Hz. Dashed lines on records indicate zero-current levels. A. Inward tail currents recorded before (Ctl) and during sequential superfusions with 2- and 10-mM Cs$^+$ solution. B. Records obtained before (Ctl) and after 20-min treatment with 30 µM 17β-estradiol. C. Comparison of the amplitudes of outward $I_{K1}$ ($-60$ mV) (left hand panel) and associated inward tail current ($-85$ mV) (right hand panel) in myocytes that were dialyzed with pipette solution that contained 0.3 mM spermine (SPM). Measurements were made at post-patch breakthrough times of 0.6 min (quasi-control) and 7 min (spermine action). † P < 0.001 versus 0.6-min amplitude (paired t test) (n = 5).
min. It reduced the amplitudes of the outward current (−50 mV) and associated tail current by 55.1 ± 3.8 and 51.3 ± 6.1% (n = 7), respectively (data not depicted). Twenty-min applications of inhibitor 17β-estradiol (30 µM) (Berger et al., 1997) had similar effects (n = 3) (e.g., Figure 22B).

The data obtained from the myocytes that were dialyzed with 0.3-mM spermine solution are shown in Figure 22C. The dialysate depressed the amplitude of outward $I_{K1}$ and concomitantly depressed the amplitude of the inward tail current. To quantify the effects, the amplitudes of outward $I_{K1}$ on depolarization to −60 mV and inward tail current on repolarization to −85 mV were measured at 0.6 and 7 min post-patch-breakthrough, and taken as quasi-control and quasi-steady-state-spermine values, respectively. Spermine dialysate decreased (P < 0.001) the amplitudes of both outward $I_{K1}$ and inward tail current by more than 80% (n = 5).

3.4.3. Effects Of Raising And Lowering $K^+$ On The Inward Tail Current

Findings in previous studies on $K^+$ accumulation in ventricular myocytes indicate that raising $K^+_o$ from 5.4 to 20 or 30 mM greatly diminishes the degree of accumulation induced by large outward $K^+$ current (Yasui et al., 1993; Clark et al., 2001). In the present study, the modulatory effects of $K^+_o$ on inward tail current were investigated by raising $K^+_o$ from 5.4 to 15 mM and by lowering $K^+_o$ from 5.4 to 2 mM.

Effects of raising $K^+_o$. Myocytes were held at a potential just negative to $E_{rev}$, and pulsed to a series of more positive potentials for 500 ms at 0.1 Hz just before and 5 min after switching from 5.4- to 15-mM $K^+$ NMDG$^+$-blocker solution. Comparison of data obtained under the two $K^+$ conditions was performed by selecting a current on a pulse to near the peak of the 5.4-mM-$K^+$ I-V relation, finding a similar-sized current in the 15-mM $K^+$ series, and measuring the amplitudes of the associated inward tail currents.
Traces of inward tail currents that followed “matched” steady-state outward currents of 1.07 nA (5.4 mM K\(^+\), −55 mV) and 1.19 nA (15 mM K\(^+\), −35 mV) are shown in Figure 23A. The amplitude of the tail current was much smaller during the superfusion with 15-mM K\(^+\) solution (−35 pA) than with 5.4-mM K\(^+\) solution (−160 pA), and its time course of decay was somewhat slower (\(\tau\) 104 ms versus 78 ms). Current amplitude data from seven experiments were normalized by expressing the amplitudes obtained during the superfusion with 15-mM K\(^+\) solution as percentages of those obtained during the superfusions with 5.4-mM K\(^+\) solution. As depicted in Figure 23B, the relative amplitudes of the (matched) outward currents were similar (98.6 ± 6.8% versus 100%), but those of the inward tails were very much smaller (26.1 ± 2.1% versus 100%).

Effects of lowering K\(^+\)_o. Myocytes were held at a potential just negative to \(E_{rev}\), and pulsed to a series of more positive potentials for 500 ms at 0.1 Hz just before and ≈ 7 min after switching from 5.4- to 2-mM K\(^+\) solution. Figure 24A shows representative records of currents elicited by pulses to potentials ≈ 30 mV positive to \(E_{rev}\). As expected from a near-square-root dependence of K1 channel conductance (\(G_{K1}\)) on K\(^+\)_o, the amplitude of the outward current was ≈ 40% smaller under the lower-K\(^+\)_o condition. Despite this reduction, the amplitude of the inward tail current was little affected (Figure 24B). In four experiments of this type, the amplitude of the outward current at −80 mV with 2 mM K\(^+\)_o was 57.5 ± 4.1% of that at −50 mV with 5.4 mM K\(^+\)_o, whereas the amplitude of the associated inward tail current near \(E_{rev}\) (−110 mV) was 113.9 ± 12.7% of that near \(E_{rev}\) (−85 mV) with 5.4 mM K\(^+\)_o. A matching of outward current amplitudes at other pulse potentials, and evaluation of associated inward tail current amplitudes, yielded 2-mM K\(^+\)_o (relative to 5.4-mM K\(^+\)_o) percentage values of 81.9 ± 5.4 and 167.4 ± 14.1% for outward current and inward tail current, respectively. Thus, when pro-rated to the amplitude of the
Figure 23. Effects of raising external K\(^+\) on the relative amplitude of the inward tail current. Myocytes sequentially bathed with 5.4-mM K\(^+\) and 15-mM K\(^+\) NMDG\(^+\)-blocker solutions were held at \(-85\) mV and pulsed (500 ms, 0.1 Hz) to potentials between \(-60\) and \(-50\) mV (5.4 mM K\(^+\)), and then held at \(-60\) mV and pulsed to potentials between \(-45\) and \(-30\) mV (15 mM K\(^+\)) for measurement of the amplitudes of end-of-pulse outward currents and associated inward tail currents. In each experiment, an outward current (15 mM K\(^+\)) was amplitude-matched with an outward current (5.4 mM K\(^+\)) for comparison of the amplitudes of the associated tail currents. 

A. Results obtained in a representative experiment. Left: the amplitudes of matched outward currents; right: the associated inward tail currents fitted with monoexponential curves. The dashed line indicates the steady-state holding current level.

B. Relative amplitudes of the 15-mM-K\(^+\) currents (5.4 mM K\(^+\) = 100%). † P < 0.001, paired t test, n = 7.
Figure 24. Effects of lowering external $K^+$ on the relative amplitude of the inward tail current. Myocytes were first bathed with 5.4-mM $K^+$ NMDG$^+$-blocker solution and held at $-85$ mV, and then bathed with 2-mM $K^+$ NMDG$^+$-blocker solution and held at $-110$ mV. A,B. Records obtained in a representative experiment. The full records are shown in A, and higher gains around the zero-current level (dashed lines) are shown in B.
outward current, the amplitude of the inward tail current in 2-mM K⁺ solution was approximately twice as large as that in 5.4-mM K⁺ solution.

3.5. **Iₚ₁ IN MYOCYTES WITH LOWERED INTRACELLULAR K⁺**

The findings below were obtained from myocytes that were bathed with solutions that contained 1 mM Cd²⁺ and 3 µM E4031. The concentration of K⁺ in the cytoplasm was lowered by dialyzing myocytes with pipette solution that contained a concentration of K⁺ lower than 140 mM. Unless otherwise specified, the osmolarity was maintained by using Cs⁺ as an equimolar replacement for K⁺.

The first section below focuses on the changes in Iₚ₁ that occurred during the early stages of dialysis with pipette solution that contained 10-20 mM K⁺. The second section is concerned with the steady-state effects of holding potential on Iₚ₁, and with the reversal potential (Eₜₚ) of Iₚ₁ in myocytes dialyzed with 0-20 mM K⁺ pipette solution. The third section details the dependence of Eₜₚ on the concentration of K⁺ in the pipette solution, and the fourth examines the interplay between Iₚ₁-mediated K⁺ flux and the concentration of K⁺ in the cytoplasm.

3.5.1. **Iₚ₁ During The Lowering Of Intracellular K⁺**

Myocytes bathed in 5.4-mM K⁺ Na⁺ solution were dialyzed with 10-mM K⁺ pipette solution to lower intracellular K⁺. Changes in Iₚ₁ occurring during this lowering were monitored by recording membrane currents in response to voltage-ramp commands, and the results obtained in a representative experiment are shown in Figure 25. The myocyte was held at −50 mV, hyperpolarized to −120 mV for 5 ms, and depolarized from there to +20 mV with 1.2-s-long voltage ramps to record I-V relations. The first ramp was applied
at 25 s post-patch-breakthrough, and subsequent ramps were applied at 15 s intervals. Records of the membrane currents elicited by a number of these ramps are superimposed on the I-V plot of Figure 25. They indicate that the I-V relation had an inwardly-rectifying shape throughout the observation period. However, there was a flattening-down of the outward-current region over this time, as well as a marked shift of the relation to more positive voltages. In addition, it was evident that shifted I-V relations “crossed over” the initial (25 s) I-V relation. Similar changes were evident in myocytes that were dialyzed with low-K⁺ NMDG⁺ pipette solution (data not shown).

The records in Figure 25 indicate that $E_{\text{rev}}$ shifted to more positive potentials as dialysis progressed. The $E_{\text{rev}}$ was $-79$ mV after 25 s of dialysis and $-45$ mV after 265 s. On the assumption that nearly all of the recorded current was contributed by $I_{K1}$ (see below), and on the additional assumption that $E_{\text{rev}} \approx E_K = -61 \log(K^+ / 5.4)$, the $E_{\text{rev}}$ values of $-79$ and $-45$ mV suggest that the concentration of K⁺ in the cytoplasm declined from $\approx 140$ mM just before patch-breakthrough, to $\approx 110$ mM after 25 s dialysis, and $\approx 30$ mM within a short time thereafter.

### 3.5.2. Effects Of Holding Potential On $E_{\text{rev}}$

The effects of holding potential on $I_{K1}$ and $E_{\text{rev}}$ in low-K⁺ myocytes are illustrated by the two sets of current traces shown in Figure 26. The upper set was recorded from a myocyte that was dialyzed with 20-mM K⁺ pipette solution and held at $-80$ mV, while the lower set was from a myocyte that was similarly dialyzed but held at $-10$ mV. The myocytes were pulsed to 0 mV for 200 ms at 0.1 Hz, and pulsing was interrupted at selected times for a series of 200-ms pulses to more positive and/or negative potentials.

Figure 26A shows records of currents elicited by pulses at 7 and 27 min post-patch-breakthrough in the myocyte held at $-80$ mV. The records indicate that there was little
Figure 25. Membrane current during the early stages of dialysis of a myocyte with 10-mM K⁺ pipette solution. The myocyte was superfused with 5.4-mM K⁺ Na⁺ solution, held at −50 mV, and probed with 1.2-s-long voltage ramps from −120 to +20 mV every 15 s. The I-V plot shows the currents elicited by selected ramps, beginning with the initial one at 25 s post-patch-breakthrough. With time after patch breakthrough, there was a slight decline in the steepness of the linear region of the inward-current limb of the I-V relation, a positive shift in the zero-current potential (E_{rev}), and a flattening of the outward-current limb.
change in the amplitudes and configurations of currents during the observation period. The currents were large and inward in direction at \(-100\) and \(-80\) mV, nearly zero at \(-60\) mV, and relatively small and outward in direction at more positive potentials. The latter outward currents featured early relaxation phases, and currents elicited by repolarizations from those test potentials (i.e., tail currents) featured early phases of increasing inward current. These types of early phases have been attributed to time-dependent development of rectification and time-dependent removal of rectification (“activation”), respectively (Matsuda & Noma, 1984; Zhang et al., 2009).

Traces of currents recorded at 11 and 62 min post-breakthrough from the myocyte held at \(-10\) mV are shown in Figure 26B. They indicate that the amplitudes and configurations of the currents were stable over this time. The currents at \(-10\) and \(-20\) mV were relatively small and outward in direction, whereas the currents at \(-40\) mV were larger and inward in direction. Thus, \(E_{\text{rev}}\) was near \(-30\) mV, or about 30 mV more positive than the \(E_{\text{rev}}\) determined in the myocyte held at \(-80\) mV.

To evaluate the extent of the modulation of \(E_{\text{rev}}\) by the holding potential, myocytes were dialyzed with 0-mM or 10-mM K\(^+\) pipette solution, and the holding potential was fixed at a level between \(-105\) and \(-25\) mV. After \(\geq 10\) min dialysis, the myocytes were pulsed with 500-ms steps to potentials above and below apparent \(E_{\text{rev}}\) before and 3-5 min after addition of 1-3 mM Ba\(^{2+}\). Actual \(E_{\text{rev}}\) values were determined using difference (raw minus Ba\(^{2+}\)) currents. The data on the plot of \(E_{\text{rev}}\) versus holding potential \((V_{\text{hold}})\) shown in Figure 27 indicate that \(E_{\text{rev}}\) was slightly more negative in the myocytes dialyzed with 10-mM K\(^+\) pipette solution (open squares) than in those dialyzed with 0-mM K\(^+\) pipette solution (filled squares), and little affected by use of NMDG\(^+\)-based pipette solution (triangles) instead of standard Cs\(^+\)-based pipette solution. However, independent of the
Figure 26. Membrane currents recorded from myocytes dialyzed with 20-mM K\(^+\) pipette solution for prolonged periods of time. The myocytes were bathed in 5.4-mM K\(^+\) NMDG\(^+\) solution, held at either \(-80\) or \(-10\) mV, and pulsed with 200-ms steps at 0.1 Hz. The dashed lines indicate zero-current levels, and times above records refer to post-patch times. A. Currents recorded from the myocyte held at \(-80\) mV. Note that \(E_{\text{rev}}\) was near \(-60\) mV. B. Currents recorded from the myocyte held at \(-10\) mV. Note that \(E_{\text{rev}}\) was positive to \(-40\) mV.
Figure 27. Dependence of the \( E_{\text{rev}} \) of \( I_{K1} \) on holding potential in low-\( K^+ \) myocytes. Myocytes bathed with 5.4-mM \( K^+ \) Na\(^+\) solution were dialyzed with 0- or 10-mM \( K^+ \) Cs\(^+\) pipette solution (pip sol’n) or with 0- or 10-mM \( K^+ \) NMDG\(^+\) pipette solution. The holding potential (\( V_{\text{hold}} \)) was constant during any given experiment, and a sequence of 500-ms steps to more negative and positive potentials was applied at \( \approx 10 \) min post-patch-breakthrough and once more at the end of a subsequent 5-min application of 1-3 mM \( Ba^{2+} \). \( E_{\text{rev}} \) values were obtained from the end-of-step I-V relations of difference (pre-\( Ba^{2+} \) minus \( Ba^{2+} \)) currents. Number of myocytes in parentheses.
dialysate used in this series of experiments, the salient feature of the data is that the more negative the holding potential, the more negative the $E_{\text{rev}}$.

The current traces in Figure 26 indicate that small outward currents are required to hold low-$K^+_i$ myocytes at $-10$ mV (and thereby secure $E_{\text{rev}}$ near $-30$ mV), and that large inward currents are required to hold them at $-80$ mV (and thereby secure $E_{\text{rev}}$ near $-60$ mV). It seemed probable that these holding currents played a role in determining $E_{\text{rev}}$, most likely by affecting the concentrations of $K^+$ across the cell membrane. A prerequisite for investigation of the role of current flow in determining $E_{\text{rev}}$ in low-$K^+_i$ myocytes was an evaluation of the dependence of $E_{\text{rev}}$ on $K^+_i$ in the absence or near-absence of either outward or inward holding current.

### 3.5.3. Effect Of Pipette $K^+$ On The $E_{\text{rev}}$ Of $I_{K1}$

The $E_{\text{rev}}$ of $I_{K1}$ was determined in experiments on myocytes that were bathed with 5.4-mM $K^+$ NMDG$^+$ solution and dialyzed with pipette solution whose concentration of $K^+$ ranged from 10 to 140 mM. In order to ensure equilibration of pipette $K^+$ and cytoplasmic $K^+$, myocytes were dialyzed for at least 15 min prior to determination of $E_{\text{rev}}$, and the magnitude of the holding current was minimized by adjustment of the holding potential.

The actual determinations of $E_{\text{rev}}$ were performed by using I-V relations generated by step commands to voltages that ranged from just below to just above zero-current voltages. The $E_{\text{rev}}$ values were as follows: $-85.3 \pm 1.1$ mV ($n = 5$) (140-mM $K^+$ pipette), $-76.6 \pm 1.4$ mV ($n = 5$) (100-mM $K^+$ pipette), $-67.2 \pm 2.6$ mV ($n = 4$) (70-mM $K^+$ pipette), $-49.1 \pm 0.9$ mV ($n = 4$) (33-mM $K^+$ pipette), $-33.2 \pm 3.3$ mV ($n = 7$) (20-mM $K^+$ pipette), and $-17.3 \pm 1.5$ mV ($n = 8$) (10-mM $K^+$ pipette). As shown in the plot of $E_{\text{rev}}$
versus the logarithm of pipette $K^+$ concentration, the data are well-fitted by an expression with slope $-59.7 \pm 1.4$ mV per decade pipette $K^+$ (Figure 28, solid line).

### 3.5.4. $I_{K1}$ And The Concentration Of Subsarcolemmal $K^+$

The pronounced dependence of the $E_{rev}$ of $I_{K1}$ on holding potential ($V_{hold}$) in low-$K^+$$_i$ myocytes (Figure 27) appeared to be related to the direction and magnitude of $I_{K1}$ at the holding potential. It seemed likely that this involved interplay between the flow of $I_{K1}$ and the concentration of $K^+$ in a submembrane cytoplasmic space of undetermined size (‘subsarcolemmal space’). Conceptually, small outward holding $I_{K1}$ mediates an efflux of $K^+$ that slightly decreases the concentration of $K^+$ in the subsarcolemmal space. Conversely, inward holding $I_{K1}$ driven, for example, by $V_{hold} << E_{rev}$, generates large resting $K^+$ influx that markedly increases subsarcolemmal $K^+$ concentration. In turn, the elevation of subsarcolemmal $K^+$ is moderated by diffusion of $K^+$ to the bulk cytoplasmic space and the pipette, as well as by a negative feedback on driving force due to the postulated negative shift in $E_K$. The results of experiments designed to investigate whether this scheme is broadly valid are described in the subsections below.

#### 3.5.4.1. Effects Of Increasing $K^+$ Influx By Increasing Inward Holding $I_{K1}$

Inward holding $I_{K1}$ was increased by increasing the inward driving force on $K^+$. The first method of doing this was to shift the holding potential from a voltage near $E_{rev}$ to one much more negative; the second was to set the holding potential just below $E_{rev}$ and then increase $K^+_o$ from 5.4 to 20 mM.

*Negative shift in holding potential.* Myocytes superfused with 5.4-mM $K^+$ $Na^+$ solution and dialyzed with 10-mM $K^+$ pipette solution were held at $-45$ mV for 10-12 min and then held at $-85$ mV for 5 min. I-V relations were determined just before and at the end of the latter period by using a series of 500-ms steps applied at 0.1 Hz. The
Figure 28. Dependence of $E_{\text{rev}}$ on the $K^+$ concentration of the pipette solution. Myocytes superfused with 5.4-mM $K^+$ $Na^+$ solution were dialyzed with pipette solution that contained a specified concentration of $K^+$ (pipette $K^+$) ($K^+ + Cs^+$ constant at 140 mM). The holding potential was kept close to the zero-current potential, and $E_{\text{rev}}$ values were determined from end-of-step (200 ms) I-V relations. The best-fit straight line to the $E_{\text{rev}}$ data ($E_{\text{rev}} = A + B \log K^+_{\text{pip}}$) has slope $B$ of $-59.7 \pm 1.4$ mV per decade pipette $K^+$) (solid line). Number of myocytes in parentheses.
change in holding potential from \(-45\) to \(-85\) mV shifted \(E_{rev}\) from \(-37.7 \pm 1.9\) mV to \(-60.6 \pm 3.6\) mV (\(n = 7\)) (\(P < 0.001\)) (Figure 29A).

In a second group of experiments, the holding potential of myocytes superfused with 5.4-mM \(K^+\) \(Na^+\) solution and dialyzed with 20-mM \(K^+\) pipette solution was set at \(-10\) mV for 10-15 min, and then shifted to \(-80\) mV for \(\approx 5\) min. I-V relations were determined by using sequences of 200-ms pulses applied just before and at the end of the latter period. In sixteen experiments, \(E_{rev}\) was \(-31.4 \pm 1.2\) mV when the holding potential was \(-10\) mV, and \(-61.0 \pm 0.8\) mV (\(P < 0.001\)) after it had been shifted to \(-80\) mV (Figure 29B). Calculations based on the assumptions that \(K^+_o\) was 5.4 mM and \(E_{rev} \approx E_K\) suggest that the concentration of intracellular \(K^+\) pertinent to \(E_{rev}\) was several millimolar lower than that in the pipette solution (20 mM) when the holding potential was \(-10\) mV, and a three-times-higher 54 mM when the holding potential was \(-80\) mV. It is possible that the inward holding current caused a depletion of \(K^+\) that affected \(E_{rev}\). However, it is important to note that even if \(K^+\) had been lowered to, say, an implausibly-low 2 mM, mean \(K^+_o\) would have been 3.7 mM, and calculated \(K^+_i \approx 37\) mM or nearly 100% larger than calculated \(K^+_i\) when the holding potential was \(-10\) mV. (The “implausible” term here is based on simulations by Pásek et al. (2003) suggesting that lowering of \(K^+\) to 2 mM in guinea-pig ventricular myocytes requires pulses to \(\approx 80\) mV negative to \(E_{rev}\)).

Increase in \(K^+_o\). When myocytes bathed in 5.4-mM \(K^+\) solution and dialyzed with 10-mM \(K^+\) pipette solution were held at \(-40\) mV for 10 min after patch-breakthrough, \(E_{rev}\) was near \(-30\) mV (e.g., Figure 27). It seemed likely that \(K^+\) influx (at \(-40\) mV) in such myocytes would be enhanced by raising \(K^+_o\) from 5.4 to 20 mM because this ought to increase both inward driving force and \(K1\) channel conductance (see Figure 4A).
Figure 29. Effects of negative shifts in holding potential on $E_{\text{rev}}$ in low-$K^+_i$ myocytes. 

A, B. Myocytes were dialyzed with 10- or 20-mM $K^+$ pipette solution and superfused with 5.4-mM $K^+$ Na$^+$ solution. The holding potential ($V_{\text{hold}}$) was set at $-45$ mV (A) or $-10$ mV (B) for 10-12 min and then shifted to $-85/-80$ mV (with the objective of increasing inward $I_{K_1}$). $E_{\text{rev}}$ values were determined from I-V relations obtained using 500-ms steps applied from $V_{\text{hold}}$ just before and 5 min after the shifts in holding potential. † $P < 0.001$, paired $t$ test. Number of myocytes in parentheses.
Myocytes dialyzed with 10-mM K\(^+\) pipette solution and held at \(-40\) mV were superfused with 5.4-mM K\(^+\) NMDG\(^+\) solution for \(\approx 15\) min, and then with 20-mM K\(^+\) NMDG\(^+\) solution for an additional 5 min. I-V relations were obtained using 2.4-s ramps from \(-160\) to \(+20\) mV every 20 s. Raising K\(^+\)\(_o\) caused a shift in holding current of \(-1025\) ± \(44\) pA, and a shift in ramp-determined E\(_{\text{rev}}\) (\(\Delta\)E\(_{\text{rev}}\)) of \(+13.5\) ± \(0.9\) mV (\(n = 7\)) (\(P < 0.001\)) (data not depicted). This \(\Delta\)E\(_{\text{rev}}\) is considerably smaller than the \(\Delta\)E\(_{\text{rev}}\) expected on the basis of \(E_{\text{rev}} \approx E_K\) where \(E_K\) is calculated on the assumption that subsarcolemmal K\(^+\) in the test myocytes is unaffected by the increase in K\(^+\)\(_o\) (i.e., in the latter case, \(\Delta\)E\(_{\text{rev}}\) \(\approx\) \(\Delta\)E\(_K\)=−61(log 5.4/20), or +35 mV). Since it seems unlikely that there was any substantial depletion of K\(^+\)\(_T\) related to large inward holding current/ramp current during the superfusions with 20 mM K\(^+\) solution, the most straightforward explanation for the observed \(\Delta\)E\(_{\text{rev}}\) of just \(+13.5\) mV is that the increase in K\(^+\) influx increased subsarcolemmal K\(^+\) concentration by at least 100\%.

3.5.4.2. Effects Of Decreasing K\(^+\) Influx By Decreasing Inward Holding I\(_{K1}\)

A number of experimental protocols were used to decrease inward holding I\(_{K1}\) in myocytes held at a potential negative to E\(_{\text{rev}}\). These included removal of external K\(^+\), application of a low concentration of Ba\(^{2+}\), and application of low concentrations of Cs\(^+\).

**Removal of external K\(^+\).** Myocytes dialyzed with 0-mM K\(^+\) pipette solution were superfused with 5.4-mM K\(^+\) Na\(^+\) solution for 12-15 min, 0-mM K\(^+\) Na\(^+\) solution for \(\approx 10\) min, and 5.4-mM K\(^+\) Na\(^+\) solution once again. During these superfusions, the myocytes were held at \(-80\) mV and pulsed to \(-40\) mV except for periodic sequences of 500-ms prepulses to \(-40\) mV followed by 500-ms test pulses to voltages between \(-85\) and \(-25\) mV. The current records obtained near the end of the initial 5.4-mM K\(^+\) superfusion indicated that E\(_{\text{rev}}\) was \(\approx -55\) mV (not shown). The current traces in Figure 30A indicate that there was little current elicited by test pulses after the switch from 5.4-mM to 0-mM
K\(^+\) solution, and robust currents after the subsequent switch to 5.4-mM K\(^+\) solution. The records also indicate that E\(_{rev}\) was near –55 mV after the latter switch.

An evaluation of whether E\(_{rev}\) was shifted to a more positive value as a consequence of inhibiting the inward holding current that was present during the initial superfusion with 5.4-mM K\(^+\) solution can be made by considering the amplitudes and directions of the currents at –80 and –40 mV during the restoration of that inward I\(_{K1}\) following the switch from 0-mM to 5.4-mM K\(^+\) solution (Figure 30B). Within 1 min of the switch to 5.4-mM K\(^+\) solution, the holding current at –80 mV had increased from approximately zero to –1.6 nA; during the same time, the test current at –40 mV had increased from approximately zero to –0.24 nA. An extrapolation of these values on an I-V plot suggests that E\(_{rev}\) at this juncture was near –25 mV. Taking E\(_{rev}\) \(\approx\) E\(_K\) = –25 mV, the subsarcolemmal concentration of K\(^+\) was \(\approx\) 14 mM provided that K\(^+\)_o had reached 5.4 mM. If K\(^+\)_o had not yet reached that level, and/or K\(^+\)_T was lower than bulk K\(^+\)_o, the subsarcolemmal K\(^+\) was lower than 14 mM. Approximately 1 min later, the current at –80 mV was \(\approx\) –1.8 nA, and the current at –40 mV was \(\approx\) zero. Thus, E\(_{rev}\) was now \(\approx\) –40 mV, and calculated subsarcolemmal K\(^+\) was \(\approx\) 25 mM assuming that mean K\(^+\)_o was 5.4 mM. At steady-state several minutes later, E\(_{rev}\) was \(\approx\) –55 mV (Figure 30B, right). On the assumption that mean K\(^+\)_o = 5.4 mM at that time, calculated subsarcolemmal K\(^+\) concentration was \(\approx\) 43 mM. In other words, the termination of K\(^+\) influx at the holding potential following the switch from initial 5.4-mM to 0-mM K\(^+\) solution resulted in a ca. 70% decrease (from 43 to 14 mM) in the concentration of subsarcolemmal K\(^+\). Similar results were obtained in five other experiments of this type.

It is important to consider whether the foregoing conclusion holds up, at least qualitatively, in the event that mean K\(^+\)_o was lower than 5.4 mM as a consequence of K\(^+\)_T
Figure 30. Effects of removal of external $K^+$ on inward holding $I_{K_1}$ and $E_{rev}$ in a low-$K^+$ myocyte. The myocyte was dialyzed with 0-mM $K^+$ pipette solution and sequentially bathed with 5.4-mM $K^+$ (not shown), 0-mM $K^+$, and 5.4-mM $K^+$ $Na^+$ solutions for $\approx 10$ min each. During this time, the myocyte was pulsed from $-80$ to $-40$ mV for 500 ms at 0.1 Hz except for determinations of I-V relations using sequences of 500-ms test pulses. A. Records obtained on I-V runs at the times marked “I-V” in panel B. The dashed line indicates the zero-current level. B. Time plot of the amplitudes of currents at $-80$ and $-40$ mV.
< 5.4 mM due to depletion (inward holding $I_{K1}$) at $-80$ mV and lack of full repletion during the prepulse to $-40$ mV and during the test pulse to $-55$ mV (see Figure 30A). Even if $K^+_T$ had been 2.6 mM (i.e., mean $K^+_o$ 4 mM), calculated $K^+_i$ at steady state was 32 mM, or more than twice the $\leq 14$ mM at 1 min.

*Application of 30 $\mu$M $Ba^{2+}$. Two low-$K^+_i$ myocytes were exposed to low $Ba^{2+}$ for 4 min to partially block K1 channels and thereby decrease inward $I_{K1}$ at a holding potential negative to $E_{rev}$. The results obtained in one of these experiments are shown in Figure 31. The myocyte was dialyzed with 10-mM $K^+$ pipette solution, pulsed from holding potential $-60$ mV to test potential $-30$ mV every 15 s, and superfused with 0-mM $K^+$ solution. After 10 min, the superfusate was changed to 5.4-mM $K^+$ solution until currents had stabilized. At that point, the 5.4-mM $K^+$ solution was supplemented with 30 $\mu$M $Ba^{2+}$ for 4 min. The $Ba^{2+}$ reduced the inward holding current at $-60$ mV by about 75%, and reduced the outward current at test potential $-30$ mV by an even greater amount (Figure 31A). A plausible explanation for this seemingly anomalous action by $Ba^{2+}$ (stronger block at a more positive voltage) is that in addition to blocking K1 channels at $-30$ mV, it caused a lowering of outward $K^+$ driving force at that potential. This view is supported by the configurations of the ramp currents recorded during the experiment (Figure 31B). The introduction of $Ba^{2+}$ shifted $E_{rev}$ from $-51$ mV to $-33$ mV. A similar result (shift of $+16$ mV) was obtained in the other experiment of this type, suggesting that inhibition of inward holding $I_{K1}$ by $Ba^{2+}$ can result in a lowering of subsarcolemmal $K^+$ via restriction of $K^+$ inflow and resultant dissipation of the intracellular $K^+$ gradient.*

*Application of Cs$. The objective of these experiments was to determine whether there is a relation between the degree of reduction of inward holding $I_{K1}$ by $I_{K1}$-blocker Cs, and the degree of shift of $E_{rev}$ in low-$K^+_i$ myocytes. In preparation for this
Figure 31. Effects of Ba\textsuperscript{2+}-induced reduction of inward holding \(I_{K1}\) on \(E_{rev}\) in a low-K\textsuperscript{+} myocyte. The myocyte was dialyzed with 10-mM K\textsuperscript{+} pipette solution and sequentially superfused with 0-mM K\textsuperscript{+} Na\textsuperscript{+} solution, 5.4-mM K\textsuperscript{+} Na\textsuperscript{+} solution, 5.4-mM K\textsuperscript{+} Na\textsuperscript{+} solution that contained 30 \(\mu\)M Ba\textsuperscript{2+}, and 5.4-mM K\textsuperscript{+} Na\textsuperscript{+} solution once again. A. Time plot of the amplitudes of currents at holding potential \(-60\) and test potential \(-30\) mV. B. Ramp I-V relations obtained at the times indicated in panel A. The Ba\textsuperscript{2+}-induced reduction of resting inward \(I_{K1}\) at \(-60\) mV (see A) contributed to an 18-mV shift in \(E_{rev}\).
determination, experiments were conducted to assess the Cs$^+$ sensitivity of $I_{K1}$ in myocytes dialyzed with 20-mM K$^+$ pipette solution and bathed with 5.4-mM K$^+$ solution. Myocytes were held at $-10$ mV and hyperpolarized to $-60$ mV for 200 ms at 0.1 Hz to elicit inward $I_{K1}$ before and during application of Cs$^+$ for 5-7 min and subsequent application of 1 mM Ba$^{2+}$ to null the current. Evaluations on eight myocytes established that (i) 1 mM Cs$^+$ had no effect on $I_{K1}$ at holding potential $-10$ mV, and (ii) inhibition of $I_{K1}$ at $-60$ mV was well-described by a Hill equation, $I_{K1} (\% \text{Ctl}) = 100/(1 + (\text{[Cs}^+\text{]/IC}_{50})^{n})$, with IC$_{50} = 0.20 \pm 0.02$ mM and Hill coefficient $n = 1.02 \pm 0.10$ (data not depicted). This IC$_{50}$ is nearly forty times lower than that determined in normal-K$^+_i$ myocytes (see Section 3.1.2 above). With this information in hand, test myocytes were dialyzed with 20-mM K$^+$ pipette solution, bathed with 5.4-mM K$^+$ solution, and held at $-80$ mV. After a 30-min equilibration period, the myocytes were treated with either 0.03, 0.1, 0.3, or 1 mM Cs$^+$ for 10-12 min, and then with 5 mM Ba$^{2+}$ to null $I_{K1}$. I-V relations were determined using 1.6-s voltage ramps from +20 mV down to $-140$ mV every 30 s.

The difference (Cs$^+$ minus Ba$^{2+}$) ramp I-V relations shown in Figure 32A illustrate that (i) the I-V relations in low-K$^+_i$ myocytes exposed to low concentrations of Cs$^+$ had inverted-bell shapes, and (ii) the voltage at maximum inward $I_{K1}$ was more positive the higher the concentration of Cs$^+$. In regard to interplay between inward holding $I_{K1}$ and subsarcolemmal K$^+$, the pertinent finding was that Cs$^+$ inhibited inward holding $I_{K1}$ and shifted $E_{rev}$ to a more positive potential. $E_{rev}$ was $-50$, $-46$, $-38$, and $-35$ mV in the myocytes treated with 0.03, 0.1, 0.3, and 1 mM Cs$^+$, respectively (Figure 32A).

The I-V relations in Figure 32A indicate that the Cs$^+$-induced rightward shift in $E_{rev}$ was coincident with a marked reduction in the amplitude of the inward holding current at $-80$ mV. Figure 32B shows a plot of $E_{rev}$ versus inward holding $I_{K1}$. The results from the
Figure 32. Effects of Cs\(^+\)-induced reduction of inward holding I\(_{\text{K1}}\) on E\(_{\text{rev}}\) in low-K\(^+\)\(_i\) myocytes. Myocytes were bathed with 5.4-mM K\(^+\) Na\(^+\) solution and dialyzed with 20-mM K\(^+\) pipette solution. A. I\(_{\text{K1}}\) and E\(_{\text{rev}}\) in four myocytes held at −80 mV, probed with 1.6-s voltage ramps from +20 to −140 mV every 30 s, treated with Cs\(^+\) for 10-12 min, and then treated with 5 mM Ba\(^{2+}\). The Cs\(^+\) – Ba\(^{2+}\) difference currents reveal that treatment with Cs\(^+\) caused positive shifts in E\(_{\text{rev}}\). B. Plot of E\(_{\text{rev}}\) versus amplitude of holding I\(_{\text{K1}}\). Open circles: Cs\(^+\) data from panel A. Filled symbols: reference data from (non-Cs\(^+\)) experiments at holding potential −80 mV (square) and −10 mV (triangle). Includes data from experiments related to Figure 29B. Crosshairs: reference intersection of E\(_{\text{rev}}\) ≈ E\(_K\) (K\(^+\)\(_i\) = 20 mM) and zero I\(_{\text{K1}}\). Number of myocytes in parentheses.
Cs⁺ experiments are represented by the open circles, and average E_{rev} and holding current amplitudes from experiments in which the holding potential was −80 mV (n = 22) or −10 mV (n = 23) are represented by the filled symbols. The overall pattern is one of a dependence of E_{rev} on the amplitude of inward holding I_{K1}.

3.5.4.3. Loss Of K⁺ From Subsarcolemmal Space

Many of the experiments described above concerned inward holding I_{K1} and putative accumulation of K⁺ in subsarcolemmal space. A question that arises is what happens to the accumulated K⁺ when the K⁺ inflow stops or is replaced by an outflow. To address this, experiments were performed in which myocytes dialyzed with 10-mM K⁺ pipette solution were held at −85 mV for ≈ 5 min to promote K⁺ inflow, and −45 mV to shut off the inflow. During this time, the current at −45 mV was monitored as test current at the end of 200-ms step depolarizations from holding potential −85 mV, and then as holding current.

The results obtained from one of these experiments are depicted in Figure 33. At the moment before the shift of the holding potential to −45 mV, the amplitude of the inward holding I_{K1} at −85 mV was approximately −2 nA (not shown) and E_{rev} was likely to have been near −60 mV (e.g., see Figure 27). Within seconds after the shift, the large K⁺ inflow at the holding potential had been replaced by a small outflow (Figure 33, open circle at ≈ 16 min) that waned over the next 30 s in a manner that most likely reflected a dissipation of the outward driving force on K⁺. The fact that the current did not simply decline in amplitude with time, but actually turned inward in direction, provides a strong indication that “extra” K⁺ in the subsarcolemmal space is drawn down not only via I_{K1}-mediated diffusion to the extracellular space, but also via diffusion to the pipette. The time course of the process was a relatively slow one; in eight experiments of this type, the halftime was 44 ± 2 s.
Figure 33. Slow equilibration and change in direction of $I_{K1}$ ($-45$ mV) following a 40-mV shift in holding potential. The myocyte was dialyzed with 10-mM K$^+$ pipette solution, superfused with 5.4-mM K$^+$ Na$^+$ solution, and sequentially held at $-85$ and $-45$ mV. The current at $-45$ mV (circles) was monitored as test current elicited by 200-ms steps from $-85$ mV or as holding current. The shift in holding potential to $-45$ mV terminated large K$^+$ influx via large inward holding $I_{K1}$ at $-85$ mV ($\approx -2.4$ nA, not shown); thereafter, outward $I_{K1}$ at $-45$ mV did not simply get smaller with time (as would be expected for outward-$I_{K1}$-mediated loss of accumulated K$^+$), but actually turned inward in direction.
3.6. DEPENDENCE OF G\textsubscript{K1} ON K\textsuperscript{+}\textsubscript{i}

3.6.1. ChordG\textsubscript{K1} Parameters During Lowerings Of K\textsuperscript{+}\textsubscript{i}

Chord conductance parameters of macroscopic K1-channel conductance (chordG\textsubscript{K1}) (see Noble, 1965; Boyett \textit{et al.}, 1980; Tourneur \textit{et al.}, 1987) were calculated from I-V relations obtained from myocytes that were dialyzed with 10-mM K\textsuperscript{+} pipette solution and held at \textasciitilde-40 mV to promote a decline in intracellular K\textsuperscript{+} and a positive shift in the E\textscript{rev} of I\textsubscript{K1}. Ramps from \textasciitilde-110 to +20 mV were applied every 15 s, with the first one at 15-30 s post-patch-breakthrough and the last one at 150-165 s post-patch-breakthrough. The amplitudes of the currents elicited by the ramps were measured at 2-mV intervals and converted to chordG\textsubscript{K1} data by dividing them by \textit{V}–E\textscript{rev}. The chordG\textsubscript{K1} data were then fitted with the Boltzmann function, chordG\textsubscript{K1} = chordG\textsubscript{K1}\textsubscript{max}/(1 + exp ((\textit{V}0.5 – \textit{V})/\textit{S})), where chordG\textsubscript{K1}\textsubscript{max} is maximal chordG\textsubscript{K1}, \textit{V}0.5 is the voltage at which chordG\textsubscript{K1} is one-half of chordG\textsubscript{K1}\textsubscript{max}, and \textit{S} is a slope factor (cf. Cohen \textit{et al.}, 1989; Missan \textit{et al.}, 2004).

Figure 34 shows results from an experiment in which the first ramp was applied at 20 s and the tenth one at 155 s. The chordG\textsubscript{K1}-V data derived from the ramp I-V relation at 155 s are shifted to the right of the data at 20 s; however, each set of data is well-described by a non-linear least-square fit of the Boltzmann function (Figure 34A). The values of the parameters obtained from fits to data from each of the ten ramps are plotted against E\textscript{rev} in Figure 34B and C. ChordG\textsubscript{K1}\textsubscript{max} declined from 92 to 75 nS, and \textit{V}0.5 from \textasciitilde-63 to \textasciitilde-44 mV, as E\textscript{rev} declined from \textasciitilde-80 to \textasciitilde-50 mV. However, slope factor \textit{S} was almost unchanged at \textasciitilde-15.5 mV (Figure 34C).

Fourteen myocytes were investigated in the manner described above. In these myocytes, E\textscript{rev} declined from \textasciitilde-79.3 ± 0.9 mV on the first ramp, to \textasciitilde-45.9 ± 1.7 mV (calculated K\textsuperscript{+}\textsubscript{i} \textasciitilde 30 mM) on the tenth ramp (P < 0.001). ChordG\textsubscript{K1}\textsubscript{max} declined from
Figure 34. ChordG$_{K1}$ parameters during the early stages of dialysis of a myocyte with 10-mM K$^+$ pipette solution. The myocyte was superfused with 5.4-mM K$^+$ NMDG$^+$ solution, held at −40 mV, and probed with 1.5-s voltage ramps at 20 s post-patch-breakthrough and every 15 s thereafter. ChordG$_{K1}$ data obtained by dividing ramp I-V data by V−E$_{rev}$ were fitted with the Boltzmann function. A. Boltzmann fits (solid lines) to chordG$_{K1}$ data calculated from the 20-s and 155-s ramp I-V data. B,C. Dependence of G$_{K1}$max, S, and V$_{0.5}$ on E$_{rev}$. 
102.5 ± 5.1 nS on the first ramp, to 87.5 ± 3.8 nS on the tenth ramp (P < 0.001) (Figure 35A), and there was a slight change in the steepness of the chordGK1-V relation (P < 0.01) (Figure 35B). V0.5 shifted from −63.6 ± 1.9 mV on the first ramp, to −44.7 ± 1.8 mV on the tenth ramp (P < 0.001) (Figure 35B). Importantly, shifts in V0.5 with time were not observed in experiments on normal-K+ i myocytes (0.5 min: −66.4 ± 2.1 mV; 10 min: −68.2 ± 2.4 mV (n = 11)).

3.6.2. ChordGK1 And Holding Potential In Low-K+ i Myocytes

To obtain further information on the relationship between GK1 and Erev in low-K+ i myocytes, chordGK1 parameters were determined in experiments in which Erev was quickly shifted by ≈ −30 mV. The myocytes were dialyzed with 20-mM K+ pipette solution and superfused with 5.4-mM K+ solution, and the shifts in Erev were achieved by moving the holding potential from −10 mV to −80 mV. I-V relations were determined just before and 5 min after the move in holding potential by using a series of 200-ms voltage steps. Erev values were obtained from polynomial interpolations of I-V data near apparent zero-current crossings, and chordGK1 data were calculated from I-V and Erev data and fitted with the Boltzmann function.

In sixteen experiments, the shift in holding potential increased Erev from −31.4 ± 1.2 mV to −61.0 ± 0.8 mV (P < 0.001), increased chordGK1max from 80.9 ± 3.5 to 90.9 ± 4.1 nS (P < 0.001), and shifted V0.5 from −46.1 ± 1.1 mV to −57.8 ± 1.5 mV (P < 0.001) (data not depicted; same myocytes as in Figure 29B).
Figure 35. Summary of changes in chordG_{K1} parameters during the early stages of dialysis of myocytes with 10-mM K^{+} pipette solution. Same experimental conditions and analysis as in Figure 34. A,B. The symbols in line with E_{rev} −80 mV and E_{rev} −45 mV indicate data obtained from first and tenth ramps, respectively, applied every 15 s (n = 14 myocytes). ** P < 0.01 and †P < 0.001 compared to first ramp, paired t test.
3.6.3. Dependence Of $\text{slope}_{G\text{K}_{1}} \text{max}$ On $E_{\text{rev}}$ And Calculated $K^+_i$

Figure 36 shows the values of $\text{slope}_{G\text{K}_{1}} \text{max}$, $E_{\text{rev}}$, and calculated $K^+_i$ obtained from experiments on 37 normal-$K^+_i$ myocytes and 83 lowered-$K^+_i$ myocytes bathed with 5.4-mM $K^+$ Na$^+$ or 5.4-mM $K^+$ NMDG$^+$ solution. The $\text{slope}_{G\text{K}_{1}} \text{max}$ values are plotted against corresponding $E_{\text{rev}}$ values in the scattergram of Figure 36A, and against the corresponding calculated-$K^+_i$ values in the scattergram of Figure 36B. The regression line in Figure 36A has slope $\text{slope}_{G\text{K}_{1}} \text{max}$ of $\approx 118$ nS at $E_{\text{rev}}$ of $-86$ mV, $\approx 109$ nS at $E_{\text{rev}}$ of $-66$ mV, $\approx 104$ nS at $E_{\text{rev}}$ of $-56$ mV, and $\approx 94$ nS at $E_{\text{rev}}$ of $-36$ mV. Thus, these results and those in Figure 36B point to moderate declines in $\text{slope}_{G\text{K}_{1}} \text{max}$ with reductions in $K^+_i$.

3.6.4. Response To Elevation Of $K^+_o$

An additional finding in regard to $G_{K1}$ in low-$K^+_i$ myocytes comes from experiments in which myocytes dialyzed with 10-mM $K^+$ pipette solution were held at $-40$ mV, bathed with 5.4-mM $K^+$ solution for $\approx 15$ min, and then bathed with 20-mM $K^+$ solution for $\approx 5$ min. In seven experiments (see also Section 3.5.4.1), $\text{slope}_{G\text{K}_{1}} \text{max}$ increased from $89.3 \pm 8.3$ to $158.9 \pm 15.1$ nS ($n = 7$) ($P < 0.001$) (data not depicted). This 1.8-fold increase in average $\text{slope}_{G\text{K}_{1}} \text{max}$ is close to the $\approx 1.92$-fold increase expected on the basis of a dependence of maximal $G_{K1}$ in mammalian ventricular myocytes on the square root of external $K^+$ concentration (Sakmann & Trube, 1984; Harvey & Ten Eick, 1988), suggesting that the dependence of $G_{K1}$ on $K^+_o$ is little affected by a lowering of $K^+_i$. 
Figure 36. Slope\(G_{K_1}^{\max}\) values determined in experiments on 37 normal-\(K_i^+\) myocytes and 83 lowered-\(K_i^+\) myocytes. A. Scattergram of slope\(G_{K_1}^{\max}\) versus \(E_{rev}\). Linear fit: \(Y = A + B \cdot X\), where \(A = 74.7\) and \(B = -0.51\). B. Scattergram of slope\(G_{K_1}^{\max}\) versus \(K_i^+\) calculated on the basis \(E_{rev} \approx E_K\). Same myocytes as in A. Linear fit: \(Y = A + B \cdot X\), where \(A = 90.3\) and \(B = 0.21\). Solid lines: linear fits of data points; long-dashed lines: 95% confidence limits of the means; short-dashed lines: 95% prediction bands.
CHAPTER 4. DISCUSSION

The results obtained in the study are discussed under five main headings: effects of lowering external K\(^+\) (Section 4.1), accumulation of K\(^+\) in restricted extracellular space (Section 4.2), effects of K\(^+\)-free bathing solution (Section 4.3), inward transients (Section 4.4), and I\(_{K1}\) in myocytes dialyzed with low-K\(^+\) pipette solution (Section 4.5).

4.1. EFFECTS OF LOWERING EXTERNAL K\(^+\)

An objective of the present study was to quantify the effects of lowering K\(^+\)\(_o\) on parameters that characterize I\(_{K1}\)-V relations. These included E\(_{rev}\), voltage at maximal amplitude of the outward limb of the relation, maximal slope of the inward limb of the relation (i.e., slopeG\(_{K1}\)max), and maximal amplitude of the outward limb of the relation. The effects of lowering K\(^+\)\(_o\) to 2 and 1 mM on these parameters are detailed below, and compared with data provided (or estimated from figures) in earlier studies.

4.1.1. Negative Shift In E\(_{rev}\)

In the experiments that examined the effects of lowering K\(^+\)\(_o\) from 5.4 mM to 2 mM, the E\(_{rev}\) determined just before the lowering was \(-85.4 \pm 1.3\) mV (n = 7). This E\(_{rev}\) is in good agreement with values determined in earlier studies on guinea-pig ventricular myocytes bathed with 5.4-mM K\(^+\) solution (e.g., Sakmann & Trube, 1984; Matsuura et al., 1987; Aronson & Nordin, 1988; Martin et al., 1995; Warren et al., 2003). The effect of lowering K\(^+\)\(_o\) to 2 mM was to shift E\(_{rev}\) to \(-111.4 \pm 1.9\) mV (n = 7). This E\(_{rev}\) can be compared with values obtained in earlier studies on the following types of ventricular
myocytes: −105 mV (guinea-pig, n = 1) (Aronson & Nordin, 1988), −109 mV (bovine, n = 1) (Isenberg & Klöckner, 1982), and (estimated) −109 ± 4 mV (cat, n = 8) (Harvey & Ten Eick, 1988). Thus, the present results on guinea-pig ventricular myocytes are in good agreement with results obtained in the earlier studies, especially those on bovine and cat ventricular myocytes.

A lowering of $K^+_o$ from 5.4 mM to 1 mM shifted $E_{rev}$ by nearly −45 mV to −129 ± 0.9 mV (n = 7). This $E_{rev}$ can be compared with two 1-mM-$K^+_o$ $E_{rev}$ values determined in earlier studies, −101 mV (n = 1) in a sheep Purkinje fibre (Dudel et al., 1967) and −127 mV (n = 1) in a rabbit ventricular myocyte (Bouchard et al., 2004) (estimated: their figure 1). Clearly, the latter value and the present one are in good agreement. Furthermore, the present one (−129 ± 0.9 mV) is indistinguishable from the $E_K$ (−130.9 mV) calculated for these myocytes. Although the $E_{rev}$ of $I_{K1}$ in guinea-pig ventricular myocytes bathed in 1-mM $K^+$ solution may be considered to be at an extremely negative voltage, it is worth noting that there are examples of even more negative ones in the literature. Current records shown by Akuzawa-Tateyama et al. (1998) indicate that $E_{rev}$ in a rabbit ventricular myocyte superfused with 0.3-mM $K^+$ solution was ≈ −145 mV (estimated; their figure 6), and $E_{rev}$ in a rabbit ventricular myocyte superfused with 0.1-mM $K^+$ solution was ≈ −155 mV (estimated; their figures 6 and 7).

4.1.2. Negative Shift In Voltage At Maximal Amplitude Of Outward $I_{K1}$

Scrutiny of figures in earlier studies on guinea-pig ventricular myocytes bathed with 5.4-mM $K^+$ solution (Sakmann & Trube, 1984; Ibarra et al., 1991; Stadnicka et al., 1997; Ishihara et al., 2002; Warren et al., 2003; Dhamoon et al., 2004; Brouillette et al., 2007) indicates that the voltage at which the amplitude of steady-state outward $I_{K1}$ reached its maximum (termed $V_{peak}$ here) was typically between −50 and −65 mV. Thus, the
“control” (5.4-mM K\(^+\)) values of V\(_{peak}\) determined in experiments on the two groups of myocytes that were subjected to superfusion with lowered-K\(^+\) solution in the present study (−54.9 ± 2.0 mV (n = 7), and −58.3 ± 1.9 mV (n = 7)) are in line with those found in earlier studies.

Lowering K\(_o\) from 5.4 mM to 2 or 1 mM shifted V\(_{peak}\) to a more negative potential. After a lowering to 2 mM, V\(_{peak}\) was −83.3 ± 2.8 mV (n = 7). Although this value is considerably more negative than that indicated in the I-V relation (2 mM K\(^+\)_o) obtained from a guinea-pig ventricular myocyte by Spindler et al. (1998) (estimated −70 mV, their figure 4A), it is in good agreement with a relation obtained from a rabbit ventricular myocyte by Shimoni et al. (1992) (estimated −84 mV, their figure 8), as well as with those obtained from guinea-pig ventricular myocytes by Aronson & Nordin (1988) (estimated −80 mV, their figure 6) and Koumi et al. (1995) (estimated −85 mV, their figure 2).

In the present study, a lowering of K\(^+\)_o from 5.4 mM to 1 mM shifted V\(_{peak}\) from −58.3 ± 1.9 to −98.3 ± 2.8 mV (n = 7). This 1-mM-K\(^+\)_o value is more negative than that indicated in the I-V relation (1 mM K\(^+\)_o) obtained from a guinea-pig ventricular myocyte by Spindler et al. (1998) (estimated −90 mV, their figure 4A), and more negative than that obtained from a rat ventricular myocyte by Bouchard et al. (2004) (estimated −92 mV, their figure 1C). However, it is less negative than that indicated in an I-V relation obtained from a rabbit ventricular myocyte by Bouchard et al. (2004) (estimated −108 mV, their figure 2).

The results obtained in the present study indicate that both V\(_{peak}\) and E\(_{rev}\) (see above) shift to more negative potentials when K\(^+\)_o is lowered from 5.4 mM. In fact, the plot of V\(_{peak}\) versus E\(_{rev}\) data in Figure 8B indicates that the two parameters change in
concert (slope 0.95 ± 0.06). Basically, this signifies that the $V_{0.5}$ of the underlying $G_{K1}$ relation shifts with $E_K$ as $K^+_{o}$ is lowered from 5.4 mM, with little change in the slope of the relation. A complementary conclusion, that the $V_{0.5}$ of the $G_{Kir}$-$V$ relation shifts with $E_K$ as $K^+_{o}$ is elevated, has been drawn from findings on strong inward-rectifier $K^+$ systems in frog skeletal muscle fibres (Hestrin, 1981) and bovine pulmonary artery endothelial cells (Silver et al., 1994).

4.1.3. Decrease In $G_{K1}$

In the present study, the magnitude of $slopeG_{K1max}$ declined to 68.2 ± 3.5% ($n = 7$) of its control 5.4-mM-$K^+_{o}$ magnitude when $K^+_{o}$ was lowered to 2 mM, and to 42.7 ± 5.7% ($n = 7$) of its control magnitude when $K^+_{o}$ was lowered to 1 mM. The only comparable data come from a study on cat ventricular myocytes by Harvey & Ten Eick (1988). In that study, $slopeG_{K1max}$ in a myocyte declined to an estimated 61% of the control 5-mM-$K^+_{o}$ value when $K^+_{o}$ was lowered to 2 mM (their figure 4B).

When the $slopeG_{K1max}$ data obtained in the present study are positioned on a double-logarithmic plot of $slopeG_{K1max}$ versus $K^+_{o}$, they are well-described by a straight line generated by the equation $slopeG_{K1max} = A\times(K^+_{o})^b$, where $b$ (slope of the line) is 0.47 ± 0.07 (Figure 8C). Thus, the dependence of $slopeG_{K1max}$ on $K^+_{o}$ over the range 5.4 to 1 mM $K^+_{o}$ is close to that of a square root one ($b = 0.50$). This finding on guinea-pig ventricular myocytes complements an earlier finding by Harvey & Ten Eick (1988). In experiments on cat ventricular myocytes, these investigators observed that the slope of the line fitting log-$slopeG_{K1max}$ versus log-$K^+_{o}$ data for $K^+_{o}$ between 2 and 20 mM was 0.56.

It is of interest to compare the present conductance data from guinea-pig ventricular myocytes with data from studies on strong inward-rectifier $K^+$ pathways in noncardiac
cells. Straight-line fits of log-slope $G_{\text{Kir}}\text{max}$ versus log-$K_o^+$ data had slopes between 0.46 and 0.55 in studies on bovine pigmented ciliary epithelial cells probed with $K_o^+$ 0.5 to 50 mM (Stelling & Jacob, 1992), RBL-1 cells probed with $K_o^+$ 5.4 to 100 mM (Wischmeyer et al., 1995), rat coronary artery smooth muscle cells probed with $K_o^+$ 6 to 140 mM (Bradley et al., 1999), bovine pulmonary artery epithelial cells probed with $K_o^+$ 6 to 120 mM (Voets et al., 1996), and Kir2.1-expressing oocytes probed with $K_o^+$ 4 to 20 mM (Kubo et al., 1993). It is also of interest that the elementary conductance of strong inward-rectifier K$^+$ channels at voltages negative to $E_K$ varies approximately with $\sqrt{K_o^+}$ for $K_o^+$ above $\approx$ 10 mM. This is indicated by the results of studies on cell-attached patches of rabbit (Kameyama et al., 1983) and guinea-pig (Sakmann & Trube, 1984; Vandenberg, 1987) ventricular myocytes, as well as by the results of studies on cell-attached patches of cultured murine macrophages (McKinney & Gallin, 1988), rabbit osteoclasts (Kelly et al., 1992), and Kir2.1-expressing host cells (Klein et al., 1999; D'Avanzo et al., 2005).

The actual mechanism whereby $K_o^+$ modulates $G_{K1}$ (and $G_{\text{Kir}}$ in noncardiac cells) remains as unclear as it was three decades ago (for example, see Leech & Stanfield, 1981; Chang et al., 2010). One proposal is that external K$^+$ ions have direct activatory action on the K$^+$ channels, most likely by occupying extracellular sites (e.g., Kubo, 1996; Murata et al., 2002) that potentially lie within the selectivity filter (Claydon et al., 2004). The other major hypothesis concerns the blocking-particle model. It holds that external K$^+$ ions bind to a high-affinity site in the channel pore, and that increases (decreases) in $K_o^+$ weaken (strengthen) pore blockade by intracellular polyvalent cations (e.g., Pennefather et al., 1992; Lopatin & Nichols, 1996; Matsuda et al., 2010).
4.1.4. Decrease In Maximal Amplitude Of Outward $I_{K1}$

It has long been observed that a several-fold lowering of $K^+_o$ from a control concentration near 5.4 mM causes a reduction in the maximal amplitude of steady-state outward $I_{K1}$ in cardiac preparations (Dudel et al., 1967; Isenberg & Klöckner, 1982; Tseng et al., 1987; Ishihara & Ehara, 1998). In the present study, a lowering of $K^+_o$ to 2 mM reduced the maximal amplitude of the outward current to $63.0 \pm 3.1\%$ ($n = 7$) of the control 5.4-mM maximal amplitude. This percentage is somewhat larger than the 51% value indicated by I-V relations obtained from a guinea-pig ventricular myocyte by Spindler et al. (1998) (their figure 4A), and somewhat smaller than that indicated by relations obtained from guinea-pig ventricular myocytes by Koumi et al. (1995) (their figure 2). However, it is close to the 60 - 68% indicated by I-V relations obtained from rabbit ventricular myocytes by Shimoni et al. (1992) (their figure 8) and Cordeiro et al. (1998) (their figure 3). In the present series of experiments in which $K^+_o$ was lowered to 1 mM, the maximal amplitude of outward $I_{K1}$ was reduced to $37.2 \pm 3.7\%$ ($n = 7$) of its control 5.4-mM $K^+$ amplitude. This value is close to the 41% value estimated from data that Spindler et al. (1998) obtained in an experiment on a guinea-pig ventricular myocyte (their figure 4A). Measurements on I-V data published by Bouchard et al. (2004) indicate that reduction of $K^+_o$ from 5 to 1 mM reduced the amplitude of maximal outward $I_{K1}$ to $\approx 54\%$ control in a rabbit ventricular myocyte (their figure 1), and to $\approx 41\%$ control in a rat ventricular myocyte (their figure 1), a value close to that found in the guinea-pig ventricular myocytes studied here.

In summary, lowering $K^+_o$ from 5.4 mM to 2 and 1 mM reduced the amplitude of maximal outward $I_{K1}$ to $63.0 \pm 3.1$ and $37.2 \pm 3.7\%$ control, respectively. When the logarithms of these values are plotted against $\log K^+_o$, they are well-fitted by a straight-line function with a slope of $0.54 \pm 0.07$ (Figure 9D). This near-$\sqrt{K^+_o}$ dependence of
maximal outward current is likely to be related to the near-$\sqrt{K^+}$ dependence of slope $G_{K1,max}$ and to a voltage dependence of steady-state rectification of $I_{K1}$ whose $V_{0.5}$ shifts with $E_K$. In a recent study on outward currents through Kir2.1 channels, Liu et al. (2011) suggested that external $K^+$ regulates the amplitude of outward currents by regulating single-channel conductance independent of changes in channel-opening probability or spermine-binding kinetics.

4.2. ACCUMULATION OF K$^+$ IN RESTRICTED EXTRACELLULAR SPACE

Yasui et al. (1993) used channel-opener nicorandil to activate $K_{ATP}$ channels in guinea pig ventricular myocytes, and found that large flows of outward $I_{K,ATP}$ were followed by inward tail currents on the repolarizations to holding potential $-85$ mV. They proposed that a depolarization-induced flow of outward $I_{K,ATP}$ causes an accumulation of $K^+$ in the T-tubules, that this accumulation shifts $E_{K,T}$ to a voltage positive to $-85$ mV, and that repolarization to $-85$ mV elicits an inward $K^+$ tail current that is likely carried by $K_{ATP}$ and K1 channels. Two central findings of Yasui et al. (1993), that flows of outward $I_{K,ATP}$ on depolarization evoke inward tail currents on repolarization, and that glibenclamide largely abolishes both of these current components, were confirmed in the present study.

Although there is good evidence that flows of outward $I_{K1}$ can cause substantial accumulations of extracellular $K^+$ in multicellular cardiac preparations (Baumgarten & Isenberg, 1977; Baumgarten et al., 1977; Boyett et al., 1980), there is seemingly no mention in the literature that they cause accumulations and evoke inward tail currents in experiments on isolated ventricular myocytes. Accumulation-related phenomena may well have been observed and judged to have been peripheral to the topic under
investigation, or not have been observed due to the use of ramps and/or repolarizations to holding potentials well below or well above the $E_{\text{rev}}$.

In the present study, myocytes were held at potentials near the $E_{\text{rev}}$ of $I_{K1}$, and repolarizations that followed depolarization-induced flows of outward $I_{K1}$ elicited inward tail currents. Both of these current components were abolished by Ba$^{2+}$. Given the experimental solutions that were used (Na$^+$-free EGTA-buffered MgATP dialysate; Na$^+$-free, glibenclamide, Cd$^{2+}$, E4031 superfusate), and the relatively low amplitude depolarizations that were applied, it is difficult to ascribe the Ba$^{2+}$-sensitive components to anything other than $I_{K1}$. A similar argument can be made in regard to concomitant reduction of outward current and inward tail current by Cs$^+$, spermine, and other K1 channel blockers.

It is important to acknowledge that while the inward tail currents are attributed to the consequences of $I_{K1}$-mediated accumulation of K$^+$ in T-tubules, there is no hard evidence that the accumulation is not occurring elsewhere. However, the following suggest that most, if not all, the accumulation occurs in the T-tubules. (i) The character of the outward-$I_{K1}$-induced inward tails resembled the character of the inward tail currents that were previously observed in ventricular myocytes, but were absent in T-tubule-sparse atrial myocytes (Yasui et al., 1993; Clark et al., 2001). (ii) Rapid-solution-change experiments on guinea-pig and other types of ventricular myocytes indicate that there is relatively slow diffusion of monovalent and divalent cations between extracellular bulk solution and extracellular solution in the T-tubules (Yao et al., 1997; Shepherd & McDonough, 1998). (iii) Mathematical simulations suggest that T-tubules should be viewed as a functional extracellular compartment in which K$^+$ can accumulate (Yasui et al., 1993; Swift et al., 2006). Finally, (iv) studies on (T-tubule-less) HEK293 cells
indicate that there is little, if any, accumulation of $K^+$ after large flows of outward $K^+$ current (Kiss et al., 1999; Frazier et al., 2000).

Yao et al. (1997) suggested that in addition to T-tubules, physical factors such as sarcolemmal folds, caveolae, and sarcolemmal-surface negatively-charged proteins might contribute to slowed diffusion of extracellular $K^+$ in heart cells. A major role for surface-sarcolemmal caveolae in the accumulation that engenders inward tail current in ventricular myocytes seems unlikely on the basis that such currents are not observed in atrial myocytes and HEK293 cells (see above). Whether caveolae in T-tubular sarcolemmal are critical for development of inward tail currents is less clear. Singer & Walsh (1980) performed calculations to evaluate whether the $K^+$ concentration of solution in caveolae is likely to differ from that in bulk solution due to $K^+$ current flow. They calculated that there would be negligible difference, primarily due to the small length of the neck of the flask-shaped caveolae (even when calculations were performed for necks elongated to an unlikely 60 nm). It could be that the situation is more complex when the caveolae are present in chainlike configurations as in the T-tubular sarcolemma of mouse ventricular myocytes; however, caveolae in the T-tubular sarcolemma of guinea-pig ventricular myocytes are almost always found as single entities (Forbes et al., 1984; Forbes & van Neil, 1988).

A consistent finding in early studies on current-flow-induced accumulation of $K^+$ in nerve and muscle preparations was that for a given-sized outward $K^+$ current, the apparent degree of accumulation was reduced by raising the concentration of $K^+$ in the bathing solution (e.g., Frankehaeuser & Hodgkin, 1956; Orkand, 1980). A similar modulation by external $K^+$ was observed in the case of accumulation in multicellular cardiac preparations (e.g., McAllister & Noble, 1966; Baumgarten & Isenberg, 1977;
Baumgarten et al., 1977). In their study on accumulation caused by outward $I_{K,\text{ATP}}$ in guinea-pig ventricular myocytes, Yasui et al. (1993) found that accumulation-related inward tail currents near $E_{\text{rev}}$ were no longer evident when 5.4-mM K$^+$ bathing solution was replaced by 30-mM K$^+$ solution. Likewise, Clark et al. (2001) observed that there was a marked reduction in the amplitude of inward tail currents induced by large K$^+$ current in mouse ventricular myocytes when external K$^+$ was raised from 5.4 to 20 mM. The results reported here are in good accord with the foregoing observations, i.e., the amplitudes of inward tails evoked after equi-sized flows of outward $I_{K1}$ were reduced by a factor of four upon replacement of 5.4-mM K$^+$ solution with 15-mM K$^+$ solution. An additional finding was that the amplitude of the tail increased by a factor or approximately two when 5.4-mM K$^+$ solution was replaced by 2-mM K$^+$ solution. The simplest explanation for the modulation of tail amplitude by K$^+$ is that outward $I_{K1,T}$ can elevate K$^+$ to a larger degree when “resting” K$^+$ ($\approx K^+_o$) is low rather than high.

An estimate of the increase in K$^+$ ($\Delta K^+_T$) induced by a 500-ms flow of outward $I_{K1,T}$ at a potential near $V_{\text{peak}}$ can be obtained by using the mean amplitude of the tail current that followed pulses to $-50$ mV in six experiments, $-169$ pA, and making the following assumptions: (i) $\Delta K^+_T$-induced changes in rectification and conductance near $E_{K,T}$ were relatively small and can be disregarded, (ii) holding potential was very close to $E_{K,b}$ (the $E_K$ determined by bathing solution K$^+$), and (iii) 50% of myocyte membrane is T-tubular membrane (Amsellem et al., 1995), and 50% of myocyte G$_{K1}$ is G$_{K1,T}$ (cf. Swift et al., 2006). Then, it can be shown that $E_{K,b} - E_{K,T}$ (or $\Delta E_K$) equals $-169$ pA/G$_{K1,T}$ (where G$_{K1,T}$ is taken as 50% of the slopeG$_{K1}$ of 69.5 nS at $E_{\text{rev}}$ measured in the six experiments). This gives $\Delta E_K = -4.86$ mV, and $\Delta K^+_T = 1.09$ mM.
4.3. EFFECTS OF K⁺-FREE BATHING SOLUTION

It has long been appreciated that switching from K⁺-containing bathing solution to K⁺-free bathing solution dramatically lowers or even nulls I_{K1} in heart cells (e.g., Dudel et al., 1967). For this reason, steady-state I-V relations determined during superfusion with K⁺-free solution have often been subtracted from those determined during superfusion with K⁺-containing solution to arrive at an improved estimate of the true I-V relation of I_{K1} at that particular K⁺_o (e.g., Dudel et al., 1967; Backx & Marban, 1993; Zaza et al., 1998; Yan et al., 2005). Statements concerning whether K⁺-free solution abolished all I_{K1} (versus “most of it”) are virtually absent in the literature. However, Backx & Marban (1993) contended that superfusion of guinea-pig ventricular myocytes with K⁺-free solution resulted in the complete elimination of I_{K1} between −100 and −30 mV. In support of this view, they reported that there was no difference in the I-V relations determined in K⁺-free solution and those determined in the presence of 1-2 mM Ba²⁺. In regard to this issue, it is worth noting that Zhang et al. (2009) stated that K⁺-free solution abolished all current carried by Kir2.1 channels in HEK293 cells.

If one takes the position that superfusion of a ventricular myocyte with K⁺-free solution completely abolishes I_{K1}, it is reasonable to suppose that the steady-state I-V relation determined during the superfusion with K⁺-free solution will be more or less inward at negative voltages due to various inward leak current components that include “background” nonselective cationic current. In fact, this is the configuration of the I-V relation most commonly presented in the literature for the K⁺-free condition at voltages between −30 and ≈ −100 mV. Examples of this configuration of I-V for guinea-pig ventricular myocytes can be found in Ishihara et al. (1989), Backx & Marban (1993), Zaza et al. (1998), Ishihara & Ehara (1998), and Song & Ochi (2002).
In the present study, the steady-state current in guinea-pig ventricular myocytes superfused with K\(^+\)-free NMDG\(^+\) solution was invariably outward at voltages as negative as \(-130\) mV, and was frequently outward at voltages as negative as \(-170\) mV. This was a most unexpected finding, and a thorough search of the literature suggested that it was a novel one but for mention in an abstract by Carmeliet (1992a) that the steady-state I-V relation in guinea-pig ventricular myocytes bathed in K\(^+\)-free Na\(^+\)-free solution (Na\(^+\) substitute not stated) and voltage-clamped using a two-suction-pipette method, was outward over the range \(-30\) to \(-120\) mV. Neither figures nor further details were provided. The steady-state outward current in the myocytes investigated here was due to outward K\(^+\) movement through K\(_1\) channels because it was absent in myocytes dialyzed with Cs\(^+\) pipette solution rather than K\(^+\) pipette solution, had an inwardly-rectifying I-V relation, and was sensitive to inhibition by concentrations of Ba\(^{2+}\) as low as 10 \(\mu\)M.

An issue that arises with the finding that the (K\(^+\)-free) steady-state I-V relation for I\(_{K1}\) is outward over a wide potential range is the level of “trace” K\(^+\) in the K\(^+\)-free NMDG\(^+\) solution. Mass spectroscopy analysis of the solution indicated levels of K\(^+\) that were below the detection limit (60 \(\mu\)M) of the system that was used (analysis performed by QE II Environmental Services, Halifax). The only reference data found in the literature concerning trace K\(^+\) in K\(^+\)-free solution come from Wang et al. (2009). They reported that NMDG\(^+\) (same supplier as used here) contributed trace K\(^+\) to K\(^+\)-free bath solution similar to that used here, such that 140-mM NMDG\(^+\) K\(^+\)-free bath solution contained \(\approx 33\) \(\mu\)M K\(^+\) (as measured using inductively coupled plasma optical emission spectroscopy).

For additional insight into the matter of trace K\(^+\) and steady-state outward I\(_{K1}\) in myocytes bathed with K\(^+\)-free solution, it is useful to turn to simulations of I\(_{K1}\)-V
relations for $K^+_o$ concentrations ranging from 5.4 mM down to 0.01 mM. The simulated relations, which are based on $E_{rev} = E_K$ and a dependence of $G_{K1}$ on $\sqrt{K^+_o}$, are shown in the log $I_{K1}$ amplitude versus voltage plot of Figure 37A. The next step in the process is to focus on the current amplitude at $-85$ mV (the standard holding potential used in the experiments with $K^+$-free solution). This is facilitated by referring to a plot of (simulated) log $I_{K1}$ amplitude at $-85$ mV, versus log $K^+_o$ for $K^+_o$ ranging from 0.008 mM to 1 mM (Figure 37B). In the figure, the amplitude of $I_{K1}$ at 1 mM $K^+_o$ (filled circle) is in line with that measured in the experiments in which bathing solution $K^+$ was elevated from nominally 0 to 1 mM (234 ± 33 pA), and the diagonal line extending from there down to the $K^+_o$ axis at 0.008 mM is in accord with the simulated I-V relations of Figure 37A. In the absence of additional information, the concentration of trace $K^+$ in $K^+$-free solution is designated as 0.03 mM. The first point to be made is that the amplitude of outward $I_{K1}$ that would be activated by the designated trace $K^+$ is $\approx 0.7$ pA in this scenario (Figure 37B, lower left), or almost two orders smaller than the experimentally measured amplitude (Figure 37B, filled diamond). Thus, according to this scenario, it is highly unlikely that trace $K^+$ in $K^+$-free bathing solution is responsible for activation of steady-state $I_{K1}$ at $-85$ mV in actual experiments.

A further item of interest in the plot of Figure 37B is the effect of addition of 0.1 mM $K^+$ on the amplitude of $I_{K1}$ at $-85$ mV. In the case of simulated $I_{K1}$, the addition increases the amplitude by $\approx 9$ pA (Figure 37B, Sim Δ); in actual experiments (e.g., Figure 9), the increase in amplitude was approximately the same ($4 \pm 3$ pA ($n = 5$)) (especially since it seems likely that the increase observed in experiments was primarily due to elevation of $K^+$ at (shell) surface membrane, rather than at T-tubular membrane (where external $K^+$ was most likely $>> 0.1$ mM (see below))). In summary, these findings
offer no support for the notion that the source of the K$^+$ that activates steady-state $I_{K1}$ ($\approx -85$ mV) in myocytes bathed with K$^+$-free solution is the trace K$^+$ in that solution. The only other K$^+$ in the experimental setting was the K$^+$ in the cytoplasm of the myocyte, and the only way that it could have activated $I_{K1}$ was to pass from the cytoplasm into an extracellular location from which it was not immediately washed away. In other words, it needed to pass into an extracellular restricted-diffusion space in which it could accumulate to a concentration much higher than that in the bathing solution. Hypothesizing that this space is that in the T-tubules appears to be reasonable in view of the evidence suggesting that K$^+$ outflow via K1 channels can accumulate there (see Chapter 3.4 and Section 4.2 above).

The situation can be visualized by reference to the diagram in Figure 38A. It shows a T-tubule, K1 channels in T-tubular membrane, and a dashed line indicating restricted diffusion of K$^+$ from T-tubular solution to bulk solution. When the myocyte is superfused with 5.4-mM K$^+$ solution and held at $V_m = -85$ mV ($\approx E_K$), the concentration of K$^+$ in the T-tubules ($K^+T$) is $\approx 5.4$ mM, and $E_K$ pertinent to T-tubular membrane ($E_{K,T}$) is the same as $E_K$ pertinent to surface membrane. When the 5.4-mM K$^+$ superfusate is exchanged for 0-mM K$^+$ superfusate, K$^+T$ declines from 5.4 mM towards 0 mM due to diffusion of K$^+$ ($J_T$) down its concentration gradient. The decline in K$^+T$ shifts $E_{K,T}$ negative to $V_m$; consequently, there is an outflow of K$^+$ ($I_{K1,T}$) from the cytoplasm to the T-tubules. As long as the cytoplasm is supplied with K$^+$ from the pipette, K$^+T$ remains higher than bulk 0-mM K$^+$. At equilibrium, the average concentration of K$^+$ in the T-tubules can be estimated by applying a series of step hyperpolarizations from $-85$ mV and determining the $E_{\text{rev}}$ of the “instantaneous” current. In the example shown in Figure 38B, $E_{\text{rev}}$ ($\approx E_{K,T}$ = $-61 \log (140/K^+_T)$) was near $-100$ mV. Overall, $E_{\text{rev}}$ was $-99.8 \pm 0.9$ mV ($n = 27$), and
Figure 37. Simulated $I_{K1}$-V relations. **A.** Plot of $I_{K1}$ amplitude versus voltage for $K^{+}_o \leq 5.4$ mM. **B.** Log-log plot of $I_{K1}$ amplitude ($-85$ mV) versus $K^{+}_o$. Filled circle: experimental mean value. Solid line: amplitude of simulated (Sim) $I_{K1}$. Vertical and horizontal dashed lines (lower left): putative trace $K^+$ (0.03 mM) in 0-mM $K^+$ solution activates simulated $I_{K1}$ of $\approx 0.7$ pA. Horizontal arrow (bottom): addition of 0.1 mM $K^+$ increases simulated $I_{K1}$ by $\approx 9$ pA (Sim $\Delta$). Open diamond: observed $I_{K1}$ in myocytes bathed with 0-mM $K^+$ solution.
Figure 38. $K_T^+$ in myocytes bathed with 0-mM $K^+$ solution and held at $-85$ mV. A. Cartoon that depicts $K^+$-related events in T-tubules when 5.4-mM $K^+$ bathing solution is replaced by 0-mM $K^+$ bathing solution. B. Determination of $E_{rev}$ in a myocyte bathed with 0-mM $K^+$ solution. The dashed line indicates the zero-current level.
calculated $K^+_T$ was near 3.2 mM. This $K^+_T$ only applies in the steady-state at $V_m = -85$ mV; it will be lower at more negative potentials. For example, hyperpolarizing pulses to $-160$ mV can elicit currents that turn outward in direction following initial inward phases; therefore, $E_{rev}$ at the time of the change in direction is $< -160$ mV, and calculated $E_{K,T}$ is $< 0.32$ mM. These matters are considered in more detail below.

4.4. INWARD TRANSIENTS

Hyperpolarizing pulses applied from holding potential $-85$ mV to voltages negative to $\approx -100$ mV elicited inward transients in myocytes bathed with $K^+$-free solution, i.e., the currents reached an inward peak shortly after the onset of the pulse, decayed in a monotonic fashion over the next several hundred milliseconds, crossed over the zero current level, and stabilized at a slightly outward level. The amplitude of the transient was dependent on the hyperpolarizing pulse that was applied: the more negative-going the pulse, the larger the amplitude of the inward peak.

The inward transient was identified as $I_{K1}$ based on the finding that it was abolished by concentrations of $Ba^{2+}$ as low as 10 µM. That being the case, the charge-carrier for the inward segment of the transient was external $K^+$. A second important finding regarding the inward transient was that it was absent in myocytes that were dialyzed with $Cs^+$ pipette solution rather than standard $K^+$ pipette solution. That absence is consistent with charge-carrier $K^+$ being localized in the T-tubules, i.e., because there was no $K^+$ flowing out across the T-tubular membrane in the $Cs^+$-dialyzed myocytes, $K^+_T$ was fully equilibrated with bulk 0-mM $K^+$, or near 0 mM. Note that if charge-carrier $K^+$ were actually in the bulk solution, its inward movement via $K1$ channels would not have been
prevented by the presence of high Cs$^+$ in the cytoplasm (see Stelling & Jacob, 1992; Matsuda, 1996).

Two additional possibilities concerning the decay phase of the inward transient deserve consideration. The first is that the decay was due to time-dependent block of inward I$_{K1}$ by external monovalent cations. Such block (see Biermans et al., 1987; Harvey & Ten Eick, 1989) has sometimes been held responsible for decay of large inward I$_{K1}$ to near-zero within several hundred milliseconds of application of a hyperpolarizing step (e.g., Ranjan et al., 1998). Since the aforementioned studies (and numerous others) indicate that external Na$^+$ (rather than NMDG$^+$) is the culprit, monovalent cation block is an unlikely cause in the present case. The second possibility is that the decay phase of the inward transient might have been caused by time-dependent block of K1 channels by trace Ba$^{2+}$ in the bathing solution (see Choe et al., 1999; So et al., 2001). However, this appears to be an unlikely explanation for the decay phase because there was little evidence of decay phases in traces of inward I$_{K1}$ in experiments on myocytes bathed with solution that contained 3 to 5.4 mM K$^+$ (e.g., Figure 1C). In other words, any trace Ba$^{2+}$ present in solutions was not an effective blocker of inward I$_{K1}$ in myocytes exposed to an external K$^+$ concentration similar to that ($\approx$ 3.2 mM) proposed to be activating I$_{K1}$ in myocytes bathed with K$^+$-free solution.

A scheme that can explain the inward transient is shown in Figure 39. The solid lines in the figure depict the effects of a hyperpolarizing pulse (from a $V_m$ of $-85$ mV to a $V_m$ of $-160$ mV) on $E_{K,T}$, on the driving force on K$^+$, and on $I_{K1,T}$. Just before the pulse, $V_m$ ($-85$ mV) is positive to $E_{K,T}$ ($\approx -100$ mV) and this drives outward $I_{K1,T}$. Early on during the pulse, $V_m$ ($-160$ mV) is negative to $E_{K,T}$ ($\approx -100$ mV) and this drives inward $I_{K,T}$. With time, the inward flow of $I_{K1,T}$ causes depletion of K$^+_T$; this reduces driving
force and attenuates inward $I_{K1,T}$. The change in direction of current occurs because concomitant diffusion of $K^+$ to the bulk solution further depletes $K_T$ and pushes $E_{K,T}$ negative to the $V_m$ of $-160$ mV. Though not shown in the figure, post-pulse return of $V_m$ to $-85$ mV should markedly increase outward driving force on $K^+$ and promote outward $I_{K1,T}$ that gradually restores $K^+_T$ and $I_{K1,T}$ to prepulse steady-state levels.

A question that arises in connection with the inward transients is whether other investigators have shown (or mentioned) them in earlier studies on ventricular myocytes.

A thorough search of the literature suggests that the answer is in the negative. Surprisingly, for all the studies that have used $K^+$-free solution as a protocol, there appears to be not one that shows records of membrane currents on hyperpolarizing pulses to potentials negative to $-100$ mV. On the other hand, there are records of membrane currents elicited by such hyperpolarizations in studies on inwardly-rectifying currents in non-cardiac cells bathed in $K^+$-free solution. Jow & Numann (1998) showed records from experiments on inwardly-rectifying $K^+$ currents in human capillary endothelial cells, and Zhang et al. (2009) showed records from experiments on inwardly-rectifying $K^+$ currents carried by Kir2.1 channels expressed HEK293 cells. In both cases, the hyperpolarizing pulses were to potentials as negative as $-120$ mV, and there were no indications of inward transients on the current records from these T-tubule-less cells.

A rough estimate of the percentage of myocyte membrane that contributes to holding $I_{K1,T}$ in myocytes bathed with $K^+$-free solution can be obtained by reference to Figure 11 where the average outward current at $15$ mV positive to $E_{rev}$ is $\approx 600$ pA under $5.4$ mM $K^+_o$ conditions, or $\approx 460$ pA at $E_{rev} + 15$ mV (i.e., at $-85$ mV) when it is scaled down (in a $K^+_o$-dependent manner) to the estimated $K^+_T$ value of $3.2$ mM. At $-85$ mV, the average amplitude of $I_{K1,T}$ was $\approx 60$ pA, or about $13\%$ of estimated $3.2$-mM whole-
Figure 39. Depiction of the effects of a ca. 300-ms hyperpolarization from −85 to −160 mV on determinants of $I_{K1,T}$. 
This suggests that holding $I_{K1}$ was contributed by $\approx 25\%$ of T-tubular membrane.

**Ramifications of findings with $K^+$-free solution.** There are a number of ramifications of the findings obtained on myocytes bathed with $K^+$-free solution. One is that whole-myocyte $I_{K1}$ should be viewed as a composite of $I_{K1}$ flowing across surface membrane ($I_{K1,S}$), and $I_{K1}$ flowing across T-tubular membrane ($I_{K1,T}$). A second arises from the fact that the flow of $I_{K1,T}$ either augments or depletes $K^+$ in the restrictive confines of the T-tubules. As a consequence, step changes in membrane potential that affect the amplitude/direction of $I_{K1,T}$ will invariably affect $K^+$ concentration in the T-tubules, and introduce extra time-dependent components into voltage-clamp recordings of whole-myocyte $I_{K1}$. These time-dependent components are likely to be at their largest in experiments on myocytes bathed in $K^+$-free solution, and at their smallest in myocytes bathed with high-$K^+$ (e.g., 30 mM) solution. They will clearly complicate analysis of time-dependent features of whole-cell $I_{K1}$ such as rectification, removal of rectification, and “inactivation”. A third ramification is that the use of $K^+$-free solution to remove interference from $I_{K1}$ in experiments on, say, delayed-rectifier $I_{Ks}$, non-selective cationic current, $Na^+$-pump current, or $Na^+-Ca^+$ exchanger current could lead to erroneous conclusions. A simple example in the case of $I_{Ks}$ is the use of (post-$I_{Ks}$-activation) hyperpolarizing pulses to a series of potentials negative to $-100$ mV in $K^+$-free solution for the purpose of determining the potential at which the $I_{Ks}$ tail reverses direction in order to evaluate the relative selectivity of the Ks channel (e.g., Hadley & Hume, 1990; Sanguinetti & Jurkiewicz, 1992); clearly, the (masked) inward transient will offset decaying outward $I_{Ks}$ tails, and make determination of the $E_{rev}$ of the $I_{Ks}$ tail seemingly impossible using this protocol.
4.5. $I_{K_1}$ IN MYOCYTES DIALYZED WITH LOW-$K^+$ PIPETTE SOLUTION

A large number of experiments were conducted on myocytes that were dialyzed with pipette solution that contained $K^+$ at a concentration lower than that in the standard 140-mM $K^+$ pipette solution. As evaluated by measurements of $E_{\text{rev}}$, the myocytes dialyzed with low-$K^+$ pipette solution had low concentrations of intracellular $K^+$ and are referred to as low-$K^+_i$ myocytes (versus normal-$K^+_i$ myocytes). The findings in experiments with low-$K^+$ pipette solution are discussed below under headings on features of $I_{K_1}$ in low-$K^+_i$ myocytes, current-flow-induced changes in $K^+_i$, and conductance and lowered $K^+_i$.

4.5.1. Features Of $I_{K_1}$ In Low-$K^+_i$ Myocytes

4.5.1.1. Dependence Of $E_{\text{rev}}$ On Pipette $K^+$

It is well established from studies on normal-$K^+_i$ ventricular preparations that raising external $K^+$ from control ca. 5 mM to a distinctly higher concentration causes a positive shift in the $E_{\text{rev}}$ of $I_{K_1}$, and that the degree of the shift in $E_{\text{rev}}$ is very close to that of the shift in $E_K$ calculated on the assumption that $K^+_i$ is unaffected by elevation of $K^+_o$ (McDonald & Trautwein, 1978; Isenberg & Klöckner, 1982; Saigusa & Matsuda, 1988). However, there is little evidence that lowering $K^+_i$ from ca. 140 mM induces commensurate positive shifts in $E_{\text{rev}}$ and calculated $E_K$. Nevertheless, it is the expected outcome based on results obtained by Hagiwara & Yoshii (1979) on inward-rectifier $K^+$ current in starfish eggs. They found that the $E_{\text{rev}}$ of the current corresponded to $E_K$ calculated on the basis that $K^+_i = \text{pipette } K^+$, or $E_{K,\text{pip}}$, when the eggs were dialyzed with pipette solutions that contained 50, 125, 200, and 300 mM $K^+$. Vandenber (1987) found that the $E_{\text{rev}}$ of average single channel current in a cell-attached patch of a guinea-pig ventricular myocyte was $\approx 10 \text{ mV}$ more negative than $E_{K,\text{pip}}$ when she dialyzed the
myocyte with a 25-mM $K^+$ internal solution via a saponin-permeabilized distal cell end. Thereafter, Saigusa & Matsuda (1988) used a patch-pipette-perfusion arrangement to exchange a 150-mM $K^+$ pipette solution for a low-$K^+$ one while the patch-pipette was sealed to a guinea-pig ventricular myocyte. They found that $E_{rev}$ deviated considerably from calculated $E_{K,pip}$. For example, the $\Delta E_{rev}$ values on switching from 150-mM $K^+$ pipette solution to 75- or 50-mM ones were < 60% of the magnitudes expected on the basis of calculated $E_{K,pip}$. They suggested that the deviation might have been due to poor exchange of dialysates in the patch-pipette.

In the present study, myocytes were dialyzed with pipette solution whose concentration of $K^+$ ranged from 10 to 140 mM, and $E_{rev}$ was determined after allowing at least 15 min for equilibration of pipette solution $K^+$ and cytoplasmic $K^+$. The overall finding was that $E_{rev}$ varied in a near-Nernstian manner with pipette $K^+$ (slope of $-59.7 \pm 1.4$ mV per decade pipette $K^+$). It seems reasonable to conclude that $E_{rev}$ has a near-Nernstian dependence on $K^+_i$.

4.5.1.2. Block By External Cs$^+$

Open-channel block by external Cs$^+$ is a signature property of Kir channels (Hille, 2001; Stanfield et al., 2002). The blocking action is quasi-instantaneous (Gay & Stanfield, 1977; Klein et al., 1999), and the degree of block increases steeply with negative potential (Hagiwara et al., 1976; Harvey & Ten Eick, 1989). An indication of the voltage dependence of block of $I_{K1}$ by Cs$^+$ is provided by the I-V relations obtained from a representative normal-$K^+_i$ myocyte before and during the application of 10 mM Cs$^+$ (Figure 3), i.e., the cation blocked $\approx 90\%$ of the current at $-100$ mV, but only $\approx 50\%$ at $-60$ mV. Overall, the $K_D$ of block at $-60$ mV in normal-$K^+_i$ myocytes was $7.5 \pm 0.3$ mM, a value in good agreement with isolated observations in an earlier study on $I_{K1}$ in cardiac muscle preparations (Trautwein & McDonald, 1978). The surprising finding was
that the $K_D$ for block at the same potential in low-$K^+_i$ myocytes was a near-40-times lower $0.20 \pm 0.02$ mM. Another way of viewing the $Cs^+$ sensitivity of $I_{K1}$ in low-$K^+_i$ myocytes compared to that in normal-$K^+_i$ myocytes is in terms of a shift in voltage dependency. In that regard, interpolation of voltage-dependent block data for 0.1 and 0.5 mM $Cs^+$ reported by Harvey & Ten Eick (1989) (their figure 3) in their study on $I_{K1}$ in normal-$K^+_i$ cat ventricular myocytes suggests that 0.2 mM was the approximate $K_D$ at $-130$ mV. In other words, the effect of lowering $K^+_i$ from $\approx 140$ mM to $\approx 20$ mM was to shift the voltage sensitivity to $Cs^+$ by $\approx +70$ mV.

The reason why $Cs^+$ is a much more potent blocker of $I_{K1}$ in low-$K^+_i$ myocytes than in normal-$K^+_i$ myocytes is not apparent. One possibility (cf., Yellen, 1984) is that the trans “knock-off” of $Cs^+$ from its binding site in the pore is severely attenuated by a decrease in $K^+_i$. In that regard, Chang et al. (2009) found that the “off” rate of $Ba^{2+}$ from the selectivity filter of the Kir2.1 channel can decline by a factor of $\approx 40$ when the concentration of $K^+$ in solution bathing the intracellular side of excised membrane patches is lowered from 200 to 20 mM.

4.5.2. Current-Flow-Induced Changes In $K^+_i$

As noted in Chapter 1, there has been considerable attention paid to intracellular accumulation (and depletion) of $Na^+$ in cardiomyocytes (for reviews, see Barry, 2006; Török, 2007), but little attention to intracellular accumulation (or depletion) of $K^+$. The most likely reason for this is that studies on $K^+$ currents in whole-cell configured myocytes are almost always conducted using pipettes that are filled with solutions that contain ca. 140 mM $K^+$, i.e., this concentration of $K^+$ in the cytoplasm, along with diffusion of $K^+$ to and from the patch-pipette, should be able to buffer gains and losses of cytoplasmic $K^+$ related to outward- and inward-directed $K^+$ current. However, the
situation is different when the myocyte is dialyzed with a solution that contains, say, 10 mM K$^+$. The “buffering” of K$^+$ flux is now very much weaker, and large flows of K$^+$ current are likely to create K$^+$ concentration gradients between submembrane cytoplasmic regions and the tip of the pipette. In regard to $I_{K1}$, the larger and the longer-lasting the current flow, the greater the putative accumulation or depletion of K$^+$ in submembrane cytoplasmic regions, and, consequently, the greater the likely discrepancy between measured $E_{rev}$ ($\approx E_K$) and calculated $E_{K,pip}$. In particular, the larger the inward flow of $I_{K1}$ generated by the driving force on K$^+$, the larger the expected discrepancy between $E_{rev}$ and $E_{K,pip}$.

In essence, this type of dependency on driving force can explain the influence of holding potential on $E_{rev}$ shown in Figure 27, i.e., the more negative the $V_{hold}$, the more negative the $E_{rev}$. A similar relationship was evident when $V_{hold}$ was changed for several minutes during the course of an experiment. Conversely, experimental manipulations (e.g., Ba$^{2+}$, Cs$^+$) that decreased K$^+$ influx by decreasing inward $I_{K1}$ at negative $V_{hold}$ shifted $E_{rev}$ to more positive potentials. In fact, there appeared to be a link between magnitude of the reduction in inward holding current and reduction of the difference between $E_{rev}$ and $E_{K,pip}$ (e.g., Figure 32).

$E_{rev}$ values in the experiments on inward holding K$^+$ current, $E_{rev}$, and $E_{K,pip}$ were used to estimate the degrees to which inward holding currents likely increased the concentration of subsarcolemmal K$^+$ above the concentration of K$^+$ in the pipette-filling solution. A complexity with these estimations was that bathing solution K$^+$ was taken as K$^+_o$ in the calculations when, in fact, effective (mean) K$^+_o$ may have been lower due to lowering of K$^+_T$ induced by the flow of inward holding current. However, as explained in connection with results obtained with a number of pertinent protocols in Chapter 3.5.4,
increases in subsarcolemmal K\(^+\) of 100% and more were calculated even when due account was taken of possible reductions in K\(^+\)_T. It is worthwhile evaluating whether the magnitudes of inward holding currents were large enough to cause large increases in subsarcolemmal K\(^+\). The E\(_{\text{rev}}\) versus holding I\(_{K1}\) data obtained from myocytes dialyzed with 20 mM K\(^+\) pipette solution (Figure 32) indicates that E\(_{\text{rev}}\) was \(\approx -60\) mV when the amplitude of the holding current was \(\approx -1.5\) nA. Thus, the amount of K\(^+\) that passed into a myocyte during a 120-s period was

\[
1,500 \text{ pA} \cdot 120 \text{ s} / 96,485 \text{ A} \cdot \text{s/mol} = 1.87 \text{ pmol}
\]

In the case of a 20-pL myocyte (Satoh et al., 1996) with osmotically-active volume-fraction of 0.67, this amount is sufficient to increase cytoplasmic K\(^+\) by 144 mM. Most of the K\(^+\) influx is likely to be counterbalanced by diffusion of K\(^+\) into the pipette. The time constant for the latter process can be estimated from the 150-165 s it takes K\(^+\)_i to fall from \(\approx 140\) mM at patch-breakthrough time to \(\approx 30\) mM (calculated from E\(_{\text{rev}}\) on tenth ramp) (Figure 34). Interpolation gives a mean \(\tau\) of 103 s (which is very close to the value (\(\approx 100\) s) calculated by Mathias et al. (1990) for the case of a 4-MΩ pipette and a 20-pL ventricular myocyte).

### 4.5.3. Conductance And Lowered K\(^+_i\)

Hagiwara & Yoshii (1979) were the first to evaluate the effects of lowering K\(^+_i\) on the conductance of strong inwardly-rectifying K\(^+\) channels (G\(_{K_{ir}}\)). That study was conducted on starfish eggs whose interiors were dialyzed using a pipette “cell-perfusion” system. In regard to chordG\(_{K_{ir}}\), these investigators found that positive shifts in E\(_{\text{rev}}\) of +24 and +53 mV induced by lowerings of K\(^+_i\) resulted in pronounced decreases in maximal conductance values. However, the shifts in E\(_{\text{rev}}\) were not accompanied by comparable
shifts in the $G_{\text{Kir}}$-V relation along the voltage axis. In fact, the relation did not shift at all in response to changes in calculated $E_K$ induced by changes in $K^+_i$, whereas it shifted in good accord with calculated $E_K$ when the latter was changed by changing $K^+_o$.

Following the work by Hagiwara & Yoshii (1979), studies on the effects of lowering $K^+_i$ on $G_{\text{Kir}}$ have been conducted on preparations as diverse as frog skeletal muscle fibres, excised *Xenopus* oocyte membrane patches containing expressed Kir2.1 channels, cell-attached patches of guinea-pig ventricular myocytes, guinea-pig ventricular myocytes, and canine Purkinje fibre myocytes. The findings obtained on the starfish eggs and the other preparations are summarized in Table 1. Also shown in the table are pertinent experimental details that include the degree of the shift in calculated $E_K$ elicited by the lowering of $K^+_i$, the method used to achieve the lowering, the external $K^+$ that was used, and the temperature at which determinations were made. Aside from the large +53-mV and +43-mV shifts achieved by Hagiwara & Yoshii (1979) and Vandenberg (1987), respectively, the $E_K$ shifts in the cited studies were relatively small (range +8.7 to +27 mV).

*Effects on maximal conductance.* Five of the eight studies reported on the effects of $E_K$ shifts on $G_{\text{Kir}}^\text{max}$. Aside from Hagiwara & Yoshii (1979) (who registered decreases of 53 and 77%), the only other study that noted decreases was that conducted on guinea-pig ventricular myocytes by Saigusa & Matsuda (1988). They reported that $G_{K1}^\text{max}$ declined by 31 and 57% for shifts in calculated $E_K$ of +8.7 and +15.4 mV, respectively.

The experimental conditions used in the present study on the effects of lowering $K^+_i$ on $G_{K1}$ differed from those in the studies cited above in that both external $K^+$ and temperature were kept at near-physiological levels. $\text{Slope } G_{K1}^\text{max}$ was measured in experiments on 120 myocytes in which $E_{\text{rev}}$ ranged from $\approx -90$ mV to $\approx -20$ mV (see
Figure 36). A synopsis of the results is that slope$G_{\text{K}1}\text{max}$ was $\approx 118$ nS at $E_{\text{rev}}$ of $-86$ mV, $\approx 109$ nS at $E_{\text{rev}}$ of $-66$ mV, $\approx 104$ nS at $E_{\text{rev}}$ of $-56$ mV, and $\approx 94$ nS at $E_{\text{rev}}$ of $-36$ mV. Importantly, a negative shift in holding potential that shifted $E_{\text{rev}}$ by an average $-30$ mV ($n = 16$ myocytes) resulted in a significant 12% increase in chord$G_{\text{K}1}\text{max}$. All in all, these results point to moderate declines in $G_{\text{K}1}\text{max}$ with reductions in $K^+$.

In that regard, they are in good agreement with the findings of Matsuda (1991), but not with those of Saigusa & Matsuda (1988).

Precisely why a lowering of $K^+_i$ may have caused a moderate lowering of $G_{\text{K}1}\text{max}$ remains to be clarified. One possibility is that a lowering of $K^+_i$ affects the blocking of K1 channels by intracellular polyvalent cations. Thus, intracellular $K^+$ ions appear to compete with channel-blocking polyvalent cations for binding sites in the pore (Lopatin & Nichols, 1996; Guo et al., 2003), and a decrease in $K^+_i$ might tip the balance in favour of blocking particles.

Amongst the experimental factors that may have contributed to the decrease in chord$G_{\text{K}1}\text{max}$ and slope$G_{\text{K}1}\text{max}$ in myocytes with lowered $K^+_i$ was the use of Cs$^+$ as replacement cation for intracellular $K^+$, i.e., intracellular Cs$^+$ ions may have exerted an inhibitory influence on inward $I_{\text{K}1}$. In that regard, Matsuda (1996) has reported that 150-mM intracellular Cs$^+$ induced flickering in K1 single-channel inward current carried by $K^+$. On the other hand, block by intracellular Cs$^+$ that affects $G_{\text{K}1}\text{max}$ is likely to be voltage-dependent and consequently would be expected to induce distortions in the shape of the $G_{\text{K}1}\text{-V}$ relation. Such distortions in the relation were not evident here (e.g., Figure 34). A second experimental factor that may have contributed to the decrease in slope$G_{\text{K}1}\text{max}$ is related to the linear section of the inward limb of the I-V relation in low-$K^+_i$ myocytes. Relative to $E_{\text{rev}}$, the linear section occurred at more negative voltages in
low-$K^{+}_{i}$ myocytes than in normal-$K^{+}_{i}$ myocytes, and this may have promoted a reduction of inward current related to depletion of $K^{+}$ in the T-tubules.

Effects on the position of the $G_{Kir}$-$V$ relation. The notations and data in the $G_{Kir}$-$V$ column of Table 1 indicate that the only studies in which shifts along the voltage axis occurred were in those conducted on guinea-pig ventricular myocytes (Saigusa & Matsuda, 1988) and canine Purkinje fibre myocytes (Cohen et al., 1989). In each of these, the $V_{0.5}$ of chord$G_{K1}$ shifted in the same direction as $E_{K}$ shifted when $K^{+}_{i}$ was lowered. The ratios of the shifts were $\approx 8/8.7$ and $15/15.4$ in the Saigusa & Matsuda (1988) study, and $\approx 5/23$ and $8/20$ in the Cohen et al. (1989) study. The results obtained in the present study, whether for smaller or larger shifts in $E_{K}$ (see Figures 34C, 35B), indicate that the ratios of the shifts (e.g., $17.8/35$, or $\approx 0.5$ (Figure 35B)) were more like those in the Cohen et al. (1989) study. A consequence of a positive shift in $V_{0.5}$ with a lowering of $K^{+}_{i}$ is that the outward-current limb of the I-$V$ relation in a low-$K^{+}_{i}$ myocyte crosses over that of a normal-$K^{+}_{i}$ myocyte. Even so, the amplitude of the outward current in the former is much smaller than that in the latter at equivalent driving forces. It may be that lowering $K^{+}_{i}$ reduces the rate of entry of intracellular $K^{+}$ ions into the pore, lowers the $K^{+}$ occupancy of the selectivity filter, and reduces $K^{+}$ exit to the extracellular solution (see Morais-Cabral et al., 2001). Inward $I_{K1}$ is far less affected by low $K^{+}_{i}$, most likely because the entry of extracellular $K^{+}$ ions into the filter and on into the vestibule is little affected, especially at sub-$E_{K}$ membrane potentials where $K^{+}$-$K^{+}$ interaction in the pore promotes efficient throughput of $K^{+}$ (see Yeh et al., 2005; Fujiwara & Kubo, 2006).
Table 1. Survey of effects of lowering $K^+_i$ on conductance parameters of strong inwardly-rectifying $K^+$ (Kir) channels.

<table>
<thead>
<tr>
<th>Study</th>
<th>$K^+_{o}$</th>
<th>Shift in $E_{rev}(E_K)$ via lowering of $K^+_i$*</th>
<th>Degree of shift in $E_K$</th>
<th>Effects of shift in $E_K$ on $G_{Kir}$ parameters</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hagiwara &amp; Yoshii (1979). Starfish egg</td>
<td>25 mM</td>
<td>+24 mV</td>
<td>↓53%</td>
<td>No shift</td>
<td>22°C</td>
</tr>
<tr>
<td></td>
<td>25 mM</td>
<td>+53 mV</td>
<td>↓77%</td>
<td>No shift</td>
<td>22°C</td>
</tr>
<tr>
<td>Leech &amp; Stanfield (1981). Skeletal muscle</td>
<td>40 mM</td>
<td>+23 mV</td>
<td>No change</td>
<td>No shift</td>
<td>3°C</td>
</tr>
<tr>
<td>Hestrin (1981). Skeletal muscle</td>
<td>50 mM</td>
<td>+26 mV</td>
<td>—</td>
<td>No shift</td>
<td>5°C</td>
</tr>
<tr>
<td>Lopatin &amp; Nichols (1996). Kir2.1 I/O patch</td>
<td>150 mM</td>
<td>+28 mV</td>
<td>—</td>
<td>No shift</td>
<td>22°C</td>
</tr>
<tr>
<td>Vandenberg (1987). Gpvm patch**</td>
<td>150 mM</td>
<td>+43 mV</td>
<td>No change</td>
<td>—</td>
<td>19°C</td>
</tr>
<tr>
<td>Matsuda (1991). Gpvm patch**</td>
<td>150 mM</td>
<td>+27 mV</td>
<td>↓7%</td>
<td>No shift</td>
<td>24°C</td>
</tr>
<tr>
<td>Cohen et al. (1989). Purkinje fibre myocyte</td>
<td>12 mM</td>
<td>+23 mV</td>
<td>—</td>
<td>Shift of +5 mV</td>
<td>9°C</td>
</tr>
<tr>
<td></td>
<td>42 mM</td>
<td>+20 mV</td>
<td>—</td>
<td>+8 mV</td>
<td></td>
</tr>
<tr>
<td>Saigusa &amp; Matsuda (1988). Gpvm</td>
<td>40 mM</td>
<td>+8.7 mV</td>
<td>↓31%</td>
<td>Shift of ≈ +8 mV</td>
<td>15°C</td>
</tr>
<tr>
<td></td>
<td>40 mM</td>
<td>+15.4 mV</td>
<td>↓57%</td>
<td>≈ +15 mV</td>
<td></td>
</tr>
</tbody>
</table>

* Lowering of $K^+_i$. Row 1, 3, 5, 6, 7, 8: via dialysis with low-$K^+$ solution. Row 2: via osmotic swelling. Row 4: via low-$K^+$ bath (internal) solution.

** Cell-attached patch on a permeabilized myocyte.

Gpvm – guinea-pig ventricular myocyte

Dashed line: data not provided.

Downward arrow: decrease of.
4.6. CONCLUDING REMARKS

Areas of the study that deserve further comment include $I_{K1}$ in relation to T-tubules, and $I_{K1}$ in myocytes with lowered cytoplasmic $K^+$ concentration.

Results obtained in the present study indicate that flows of outward $I_{K1}$ elicited by step depolarizations can cause accumulation of $K^+$ in the T-tubules of guinea-pig ventricular myocytes. The accumulation introduces extra time-dependent components into voltage-clamp recordings of whole-myocyte $I_{K1}$. These time-dependent components are more pronounced under low-$K^+_{o}$ than high-$K^+_{o}$ conditions, and are likely to complicate analysis of time-dependent features of $I_{K1}$ such as rectification and removal of rectification, as well as analysis of other membrane currents recorded in the presence of $I_{K1}$. During cardiac electrical activity, accumulation related to $I_{K1,T}$ flowing during the latter phases of repolarization may well persist into early diastole and influence cell excitability. In turn, accumulation and its influence on excitability are likely to be affected by a wide range of physiological and pathophysiological factors, including heart rate, extracellular $K^+$ concentration, and status of $K1$-channel conductance, not to mention T-tubule remodelling/dysfunction (Louch et al., 2010; Ibrahim et al., 2011).

Bearing in mind the likely central physiological role of T-tubules in myocyte $K^+$ movement and cardiac electrical activity, it will be important to extend this area of study in two directions. The first of these would address the question of whether membrane currents in guinea-pig ventricular myocytes subjected to detubulation procedures behaved in a manner consistent with signature findings in the present work. The second would be to develop a theoretical model to evaluate the importance of T-tubular geometry and T-tubular $K^+$ currents on myocyte electrical activity.
In regard to $I_{K1}$ in myocytes with lowered cytoplasmic $K^+$ concentration, results obtained on myocytes dialyzed with < 140-mM $K^+$ pipette solution indicate that the dependence of $E_{rev}$ on log pipette $K^+$ could be well-fitted by a straight line with slope $-59.7 \pm 1.4$ mV per decade of pipette $K^+$ concentration. It seems reasonable to conclude that $E_{rev}$ has a near-Nernstian dependence on $K^+_i$. A ramification of this result is that provided $G_{K1}$ near $E_K$ remains relatively strong, and resting inward cationic and anionic conductances relatively small, $K^+_i$ is almost certainly a major determinant of the resting potential of ventricular myocytes as previously suggested by Baumgarten & Fozzard (1992).
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