Involvement of anion channel(s) in the modulation of the transient outward K⁺ channel in rat ventricular myocytes

Xiao-Gang Lai,† Jun Yang, Shi-Sheng Zhou,† Jun Zhu, Gui-Rong Li, and Tak-Ming Wong

1Department of Physiology, The Fourth Military Medical University, Xi’an 710032; 2Institute of Basic Medical Sciences, Medical College, Dalian University, Dalian 116622; and 3Faculty of Medicine, Department of Physiology, The University of Hong Kong, Hong Kong, China

Submitted 11 July 2003; accepted in final form 17 February 2004

Lai, Xiao-Gang, Jun Yang, Shi-Sheng Zhou, Jun Zhu, Gui-Rong Li, and Tak-Ming Wong. Involvement of anion channel(s) in the modulation of the transient outward K⁺ channel in rat ventricular myocytes. Am J Physiol Cell Physiol 287: C163–C170, 2004. First published February 18, 2004; 10.1152/ajpcell.00297.2003.—The cardiac Ca²⁺-independent transient outward K⁺ current (Iₒ), a major repolarizing ionic current, is markedly affected by Cl⁻ substitution and anion channel blockers. We reexplored the mechanism of the action of anions on Iₒ by using whole cell patch-clamp in single isolated rat cardiac ventricular myocytes. The transient outward current was sensitive to blockade by 4-aminopyridine (4-AP) and was abolished by Cs⁺ substitution for intracellular K⁺. Replacement of most of the extracellular Cl⁻ with less permeant anions, aspartate (Asp⁻) and glutamate (Glu⁻), markedly suppressed the current. Removal of external Na⁺ or stabilization of F-actin with phalloidin did not significantly affect the inhibitory action of less permeant anions on Iₒ. In contrast, the permeant Cl⁻ substitute Br⁻ did not markedly affect the current, whereas F⁻ substitution for Cl⁻ induced a slight inhibition. The Iₒ elicited during Br⁻ substitution for Cl⁻ was also sensitive to blockade by 4-AP. The ability of Cl⁻ substitutes to induce rightward shifts of the steady-state inactivation curve of Iₒ was in the following sequence: NO₃⁻ > Cl⁻ > Br⁻ > gluconate⁻ > Glu⁻ > Asp⁻. Depolymerization of actin filaments with cytochalasin D (CytD) induced an effect on the steady-state inactivation of Iₒ similar to that of less permeant anions. Fluorescent phalloidin staining experiments revealed that CytD pretreatment significantly decreased the intensity of FITC-phalloidin staining of F-actin, whereas Asp⁻ substitution for Cl⁻ was without significant effect on the intensity. These results suggest that the Iₒ channel is modulated by anion channel(s), in which the actin cytoskeleton may be implicated.

DEPOLARIZATION-ACTIVATED OUTWARD K⁺ CURRENTS

Depolarization-activated outward K⁺ currents play important roles in the regulation of the action potential plateau and duration in many mammalian hearts (3, 4). Two basic types of depolarization-activated outward K⁺ currents have been distinguished on the basis of differing time- and voltage-dependent properties and pharmacological sensitivities. One is a rapidly activating and inactivating transient outward current (Iₒ), which is sensitive to 4-aminopyridine (4-AP). The other is a slowly activating, tetraethylammonium-sensitive delayed-rectifier K⁺ current (3, 4). In rat cardiac ventricular myocytes, the Iₒ is a 4-AP-sensitive and intracellular Ca²⁺ (Ca⁺⁺)-independent K⁺ current (3, 14, 22), which is referred to as Iₒ in the present study. Considerable evidence suggests that the voltage-gated K⁺ channels Kv4.2 and Kv4.3 contribute to cardiac Iₒ (35).

Initial studies found that the Iₒ was reduced in Cl⁻-free solution and concluded that this current was carried primarily by Cl⁻ (13, 15, 18). However, later studies revealed that K⁺, rather than Cl⁻, was the main charge carrier of the current (24, 25). Inhibition of the current induced by less permeant Cl⁻ substitutes was thought to be probably the result of a decrease in free Ca²⁺ concentration, since the Cl⁻ substitutes used in those studies might chelate external Ca²⁺ (Ca⁺⁺); see Ref. 23. This notion was supported by the observation that the Iₒ was less sensitive to replacement of external Cl⁻ (Clₒ) when free Ca²⁺ was kept at a constant level (24, 25). Further studies also provided some evidence for the dependence of the Iₒ on Ca²⁺. Reducing Ca²⁺ by EGTA inhibits the Iₒ (41). On the other hand, other studies showed that the current in rat cardiac ventricular myocytes displays a Ca²⁺-independent property (3, 22), which is also inhibited by less permeant Cl⁻ substitutes and anion channel blockers (ACB; see Ref. 30). Heretofore, the mechanism of the influence of less-permeant Cl⁻ substitutes on cardiac Iₒ remains unclear.

In the mammalian heart, several anion channels have been functionally identified for over a decade (19). In the study of cardiac anion channels, a common phenomenon is that Cl⁻ substitutes and ACB have profound effects on other channels (1, 8, 16, 31, 43, 44), which is generally attributed to nonspecific effects. However, our primary study suggests that the nonspecific effects of Cl⁻ substitutes and ACB on cardiac Ca²⁺ channel may implicate a channel-channel interaction (46), an important regulating mode found in epithelia (27, 40). It is well accepted that the cystic fibrosis transmembrane conductance regulator (CFTR) may act both as a Cl⁻ channel and a regulator of the activity of other epithelial channels via a channel-channel interaction (27, 40), possibly mediated by the cytoskeleton (20). Moreover, studies have found that a variety of channels are regulated by the actin cytoskeleton (2, 7, 17, 20, 33, 34, 36, 45). The Kv4.2, which contributes to the Iₒ, is found to interact with filamin, a member of the α-actinin/ spectrin/dystrophin family of actin-binding proteins. Absence of filamin results in suppression of Kv4.2 current (36). In mammalian cardiac ventricular myocyte, there is a possibility that one channel activity may influence other channel(s) via the actin cytoskeleton. Therefore, in the present study, to evaluate the hypothesis that channel-channel interaction might be implicated in the action of Cl⁻ substitutes, we reexamined the
effects of Cl\(^-\) substitution on \(I_{\text{so}}\). The present data indicate that the effects of anions on \(I_{\text{so}}\) are closely related to their permeability, and disruption of the actin microfilament by cytochalasin D (CytD) produced an effect similar to that of less permeant anions on the steady-state inactivation of \(I_{\text{so}}\). The present results provide evidence for the hypothesis that the effects of anions on \(I_{\text{so}}\) involve a modulatory action of anion channel(s) on \(I_{\text{so}}\) channel, probably mediated by the actin cytoskeleton.

**MATERIALS AND METHODS**

**Cell preparations.** Ventricular myocytes were enzymatically isolated from adult Sprague-Dawley rats (200–250 g), as reported previously (46). Briefly, the hearts were removed immediately after decapitation and retrogradely perfused at 37°C with the following solutions in turn: Tyrode solution (5 min), Ca\(^{2+}\)-free Tyrode solution (5 min), Ca\(^{2+}\)-free Tyrode solution with 0.5 mg/ml collagenase (type 1; Sigma, St. Louis, MO) and 1 mg/ml BSA (35 min), and Kraftbrühe (KB; high K\(^+\)) solution (5 min). After dissociation and collection, the cells were kept in KB solution at room temperature (23–25°C) for electrophysiological recordings. To study the role of the actin cytoskeleton, myocytes were incubated with either 100 µM CytD and 100 µM phalloidin or vehicle (DMSO) at 37°C using a patch-clamp amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). The offset potentials between both electrodes were zeroed before the pipette touched the cell. The liquid junction potential between the Asp\(^-\)/AgCl pipette and the standard bath solutions for recording \(I_{\text{so}}\), was calculated to be 15 mV by using the JPCalc program within Clampex 8.1 (Axon Instruments) and was corrected after the experiments. To minimize changes in liquid junction potentials caused by alteration in Cl\(^-\), the Ag-AspCl bath ground electrode was placed in a separate pool of 3 M KCl, which was connected to the recording chamber by a 3 M KCl-agar bridge, as reported previously by Zygmunt and Gibbons (47). Whole cell \(I_{\text{so}}\) was elicited by 300-ms pulses from a holding potential of −65 mV (after correction of the junction potential) to test potentials ranging from −55 to +45 mV in 10-mV increments. Whole cell basal Cl\(^-\) currents were elicited from a holding potential of −40 mV to test potentials ranging from −100 to +100 mV in 20-mV increments. The pulses were 200 ms in duration and delivered at 1-s interval. The current signals were low-pass filtered at 5 kHz and stored in the hard disk of an IBM-compatible computer. \(I_{\text{so}}\) was calculated by subtracting the peak outward current from the current at the end of the test pulse.

**Confocal imaging.** Fluorescence labeling of F-actin was performed as previously described (29). Cell suspensions, pretreated with different protocols, were sedimented by centrifugation at 100 g for 1 min. The supernatant was discarded. Cells were then fixed in 4% paraformaldehyde-PBS solution for 10 min and washed three times with PBS by centrifugation. Cell suspensions were transferred to slides and kept in 4°C overnight. The cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After three washes with PBS, cells were stained with FITC-labeled phalloidin (5 µM in PBS) to localize F-actin for 50 min in a dark room at room temperature and then washed extensively with PBS. Slides were visualized using an Olympus Fluoview FV300 confocal microscope. FITC was excited at 490 nm and detected at 520 nm. Optical sections (0.6 µm thick) were taken of each sample to eliminate out-of-focus fluorescence of the intensely stained myocytes. To standardize the fluorescence intensity for all the experimental preparations, the time of image capture, the image intensity gain, the image enhancement, and the contrast and brightness settings were optimally adjusted at the outset and kept constant for all experiments.

**Solutions.** The Tyrode solution contained (in mM) 143 NaCl, 5.4 KCl, 0.5 MgCl\(_2\), 1.8 CaCl\(_2\), 0.3 NaH\(_2\)PO\(_4\), 5 glucose, and 5 HEPES-NaOH (pH 7.4). The nominally Ca\(^{2+}\)-free Tyrode solution was made by omitting CaCl\(_2\) from the normal solution. The KB solution contained (in mM) 70 potassium glutamate, 25 KCl, 20 taunine, 10 KH\(_2\)PO\(_4\), 3 MgCl\(_2\), 0.5 EGTA, 10 glucose, and 10 HEPES-KOH (pH 7.5).

The pipette solution for recording \(I_{\text{so}}\) contained (in mM) 110 potassium aspartate (unless specifically stated in the text), 20 KCl, 1 MgCl\(_2\), 5 Na\(_2\)-phosphocreatine, 0.1 GTP, 5 MgATP, 5 EGTA, and 10 HEPES (pH was adjusted to 7.2 with KOH). In some experiments, intracellular K\(^+\) (K\(^+\)) 130 mM was replaced by equimolar Cs\(^+\) (pH = 7.2, adjusted with CsOH). The standard bath solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl\(_2\), 0.4 K.H\(_2\)PO\(_4\), 1.8 CaCl\(_2\), 1 BaCl\(_2\), 0.5 CdCl\(_2\), 5 HEPES, and 10 glucose (pH 7.4). BaCl\(_2\) and CdCl\(_2\) were used to inhibit the inwardly rectifying K\(^+\) current, the L-type Ca\(^{2+}\) current, and the Ca\(^{2+}\)-activated Cl\(^-\) current (48). In some experiments, external Na\(^+\) (Na\(^+\)) was replaced by equimolar N-methyl-D-glucamine (NMDG). In Cl\(^-\) substitution experiments, 140 mM Cl\(^-\) was replaced by equimolar Br\(^-\), I\(^-\), NO\(_3\)-, aspartate (Asp\(^-\)), glutamate (Glu\(^-\)), or gluconate (Glc\(^-\)). The free Ca\(^{2+}\) concentration in bath solutions was calculated using the CalBus program (provided by Dr. G. Droogmans, Katholieke Universiteit Leuven, Belgium), as described previously (46). According to the calculation, the free Ca\(^{2+}\) in the bathing solutions after substitution of 140 mM Cl\(^-\) with equimolar Asp\(^-\), Glu\(^-\), or Glc\(^-\) was 1.76, 1.78, and 0.57 mM, respectively, when the total Ca\(^{2+}\) was 1.8 mM. Because the presence of other bivalent cations, Ca\(^{2+}\) and Ba\(^{2+}\), could not be taken into account in the calculation, the free Ca\(^{2+}\) concentration after replacement of Cl\(^-\) with less permeant anions may represent an underestimate.

The solutions for recording basal Cl\(^-\) current were as follows. The pipette solution contained (in mM) 135 NMDG-Cl, 2 EGTA, 5 Mg-ATP, 10 HEPES, and 10 mannitol, pH 7.2, with intracellular Cl\(^-\) (Cl\(^-\))(\(s\)) = 135 mM. The standard bath solution contained (in mM) 125 NaCl, 2.5 MgCl\(_2\), 2.5 CaCl\(_2\), 5 glucose, 10 HEPES, and 30 mannitol, pH 7.4, with Cl\(^-\)(\(b\)) = 135 mM. Nifedipine (1 µM) was added to the bath solution to inhibit the L-type Ca\(^{2+}\) current. The membrane potential was depolarized from −70 to −40 mV, where it was held for 100 ms to inactivate the Na\(^+\) channels. K\(^+\) currents were eliminated by omission of K\(^+\) from pipette and bath solutions. In some experiments, 125 mM Cl\(^-\) was replaced by either equimolar Br\(^-\) or Asp\(^-\). Chemicals. 4-AP, nifedipine, CytD, phalloidin, and FITC-phalloidin were purchased from Sigma. Stock solutions of CytD (50 mM) and phalloidin (50 mM) in DMSO were diluted to the desired final concentrations immediately before use.

**Statistical analysis.** Data are presented as means ± SE. Statistical differences in the data were evaluated by Student's t-test or ANOVA as appropriate and were considered significant at values of \(P < 0.05\). **RESULTS**

**Effect of less permeant anions on \(I_{\text{so}}\).** The \(I_{\text{so}}\), recorded in rat ventricular myocytes, was inhibited either by the specific \(I_{\text{so}}\) channel blocker 4-AP (\(n = 5\), data not shown) or by Cs\(^+\) substitution for K\(^+\) (\(n = 4\), data not shown), indicating that the current is the 4-AP-sensitive and Ca\(^{2+}\)-independent \(I_{\text{so}}\), as previously reported (3, 22). To explore the mechanism of action of Cl\(^-\) substitutes on \(I_{\text{so}}\), we first observed the effects of replacing Cl\(^-\) with less permeant anions, Asp\(^-\) and Glu\(^-\). Figure 1 shows the effects of substituting most of the Cl\(^-\) (140 mM) with equimolar Asp\(^-\) or Glu\(^-\) on \(I_{\text{so}}\). In Na\(^+\)-rich bath solution (Na\(^+\)(\(b\)) = 140 mM), substitution of Cl\(^-\) by Asp\(^-\)
inhibited $I_o$ (Fig. 1A), without discernible change in cell volume or morphology under the light microscope. The inhibition was $85.1 \pm 3.9\%$ at $+45$ mV ($n = 9, P < 0.01$). Similar effects were observed with Glu substitution for Cl (Fig. 1B). Replacement of Cl with Glu decreased the peak $I_o$ at $+45$ mV by $57.7 \pm 4.3\%$ ($n = 7, P < 0.01$). Substitution of Cl by Asp or Glu did not significantly affect the sustained component of outward K current (Fig. 1). To examine whether Na+K+-2Cl-cotransport is involved in the inhibition of less permeant anions, we observed the effects of less permeant Cl substitutes on the $I_o$ activated in 0 mM Na+ (replacement of Na+ with the impermeant monovalent cation NMDG+). Perfusing the cells with Na+-free solution did not prevent the inhibitory effect of the less permeant Cl substitutes Asp (Fig. 2A) or Glu (Fig. 2B) on $I_o$, indicating that Na+K+-2Cl-cotransport does not play a crucial role in this inhibitory effect. These results suggest that the regulation of $I_o$ involves anion-related factor(s).

Effects of permeant anions on $I_o$. We then determined the effect of the permeable anions, F- and Br-, on $I_o$. Replacement of most of Cl with F- reduced the current slightly (Fig. 3A) compared with Asp- or Glu- substitution (Fig. 1). The inhibition by F- substitution for Cl was $23.5 \pm 3.6\%$ at $+45$ mV, which was statistically significant ($P < 0.01, n = 5$). Replacement of the majority of Cl with equimolar Br- also seemed to reduce $I_o$ (Fig. 3B). At $+45$ mV the inhibition was $8.8 \pm 3\%$ ($n = 6$), which was not significant statistically. The $I_o$ elicited by Br- substitution was also sensitive to blockade by 4-AP (Fig. 3C, $n = 4$). The effects of a variety of anions on $I_o$ are summarized in Fig. 4. The facilitative effect of different anions on the activation of $I_o$ was in the following sequence: NO3- > Cl- > Br- > F- ≈ Glu- > Glu- > Asp-.
These results indicate that the activation of $I_{to}$ is closely related to anionic permeability, i.e., $I_{to}$ is more easily activated with increasing permeability of external anion. Thus it is likely that anionic permeability dependence of $I_{to}$ may involve the activity of anion channel.

**Effect of different anions on the steady-state inactivation of $I_{to}$**

To explore the mechanisms of action of Cl$^-$/H11002 substitutes on $I_{to}$, we measured the voltage dependence of the steady-state inactivation of $I_{to}$ in the Cl$^-$/H11002 substitution conditions. The potential at which the current was completely inactivated shifted to a more positive potential (from −35 to −25 mV) after replacement of Cl$^-$/H11002 with NO$^-_3$ (Fig. 5A). In contrast, after replacement of Cl$^-$/H11002 with Asp$^-$/H11002, the current was completely inactivated at a much more negative potential (−55 mV) than in control (−25 mV; Fig. 5B). Figure 5C shows the effects of a variety of anions on the steady-state inactivation-voltage relationships of $I_{to}$. NO$^-_3$ substitution for Cl$^-$/H11002 caused a shift in the potential of 50% inactivation of $I_{to}$ ($V_{0.5}$) toward a more positive potential.

**Fig. 3. Effects of substitution of Cl$^-$/H11002 with relatively permeant anions on cardiac $I_{to}$.**

$I_{to}$ was elicited with the protocol as described in Fig. 1. A: representative current traces recorded before (a) and after (b) 140 mM Cl$^-$/H11002 in the standard bath solution was replaced by equimolar F$^-$ in the presence of 4 mM 4-aminopyridine (4-AP) in Br$^-$/H11002 substitution (c), and after withdrawal of 4-AP (d).

**Fig. 4. Effects of Cl$^-$/H11002 substitutes on the current-voltage relationship of the $I_{to}$.** The currents were elicited in the Na$^+$-rich bath solution (Na$^+$ = 140 mM) by applying 300-ms depolarizing pulses from a holding potential of −65 mV in 10-mV increments, between −55 and +45 mV before (control) and after substitution of 140 mM Cl$^-$/H11002 with NO$^-_3$ (n = 6), Br$^-$ (n = 6), F$^-$ (n = 5), Glc$^-$ (n = 7), Glu$^-$ (n = 8), or Asp$^-$ (n = 9). For each cell, $I_{to}$ was measured by subtracting the peak outward current from the sustained current at each test potential and normalized to their respective amplitudes evoked at +45 mV in control (Cl$^-$/H11002 = 140 mM).

**Fig. 5. Effects of Cl$^-$/H11002 substitutes on the steady-state inactivation of $I_{to}$ in rat ventricular myocytes.** Original current traces recorded before (A and B, left) and after replacement of the majority of Cl$^-$/H11002 (140 mM) with NO$^-_3$ and Asp$^-$/H11002 in A and B, right, respectively. $I_{to}$ was activated by 300-ms test pulses to +45 mV from holding potentials ranging from −125 to −15 mV in 10-mV increments. Nos. in A and B indicate the membrane potentials at which the currents were recorded. C: effects of Cl$^-$/H11002 substitutes on the steady-state inactivation curve of $I_{to}$. Currents in C were expressed as fractions of maximal $I_{to}$ (H$_{max}$) and plotted as a function of voltage. Curves were fitted to experimental data using the Boltzmann equation: $I/I_{max} = 1/[1 + \exp((V_{m} - V_{0.5})/k)]$, where $V_{0.5}$ and $k$ are the potentials of half-maximal inactivation and the slope factor, respectively.
positive potential by 5.7 ± 1.1 mV ($P < 0.01, n = 7$). In contrast, replacement of Cl\textsuperscript{−} with Glc\textsuperscript{−}, Glu\textsuperscript{−}, or Asp\textsuperscript{−} shifted the $V_{0.5}$ toward a more negative potential by 5.2 ± 0.9 mV ($P < 0.01, n = 6$), 12.1 ± 0.8 mV ($P < 0.01, n = 5$), and 23.2 ± 1.8 mV ($P < 0.01, n = 8$), respectively. Br\textsuperscript{−} substitution for Cl\textsuperscript{−} did not significantly influence the steady-state inactivation curve of $I_{\text{no}}$ ($n = 6$). The ability of various anions to shift the steady-state inactivation curve of $I_{\text{no}}$ to more positive potentials was in the following sequence: NO\textsubscript{3}\textsuperscript{−} > Cl\textsuperscript{−} > Glc\textsuperscript{−} > Glu\textsuperscript{−} > Asp\textsuperscript{−}.

Effect of CytD treatment on the steady-state inactivation of $I_{\text{no}}$. Both voltage-gated K\textsuperscript{+} channels (17, 33, 34, 36) and anion channels (e.g., the CIC-2 Cl\textsuperscript{−} channel, the CFTR channel, and the swelling-activated Cl\textsuperscript{−} channel; see Refs. 2, 7, and 45) are linked to the actin cytoskeleton and are regulated by it. Because the effects of anions on $I_{\text{no}}$ may involve an interaction between the $I_{\text{no}}$ channel and anion channel(s), we investigated whether the actin cytoskeleton was involved in the actions of anions by disrupting actin microfilaments with CytD, a fungal toxin known to specifically break down F-actin fibers (9). In DMSO treatment myocytes, substitution of Cl\textsuperscript{−} with Asp\textsuperscript{−} caused a 19.5 ± 1.9 mV leftward shift of the $V_{0.5}$ of $I_{\text{no}}$ ($n = 19, P < 0.01$). In contrast, the same substitution in CytD-treated myocytes only caused a 7.4 ± 1.4 mV leftward shift of the $V_{0.5}$ ($n = 25$). In some CytD-pretreated myocytes (4 of 25 cells), replacement of Cl\textsuperscript{−} with Asp\textsuperscript{−} did not produce significant shift of the $I_{\text{no}}$ inactivation curve (Fig. 6, B and C). These data indicate that disruption of actin microfilaments produces effects mirroring those less permeant anions, suggesting that the effects of anions on the $I_{\text{no}}$ channel may involve the actin cytoskeleton.

Imaging of F-actin in myocytes pretreated either with Asp\textsuperscript{−} or with CytD. To assess the possibility that less permeant Cl\textsuperscript{−} substitutes may induce F-actin disruption, we observed the structural changes in the actin cytoskeleton of cardiac myocytes pretreated with Asp\textsuperscript{−}-rich external solution. Confocal images showed that cells pretreated with Asp\textsuperscript{−}-rich external solution did not induce significant changes in the average pixel intensity of FITC-phalloidin staining of F-actin (Fig. 7B) compared with the images of myocytes pretreated with control solution (Fig. 7A). In contrast, CytD pretreatment induced a significant decrease in the FITC-phalloidin staining intensity of F-actin (Fig. 7D) compared with the control cells treated only with the vehicle (Fig. 7C). Moreover, stabilization of actin cytoskeleton with phalloidin did not prevent Asp\textsuperscript{−} substitution-induced inhibition of $I_{\text{no}}$ (Fig. 6A, $n = 4$). These results indicate that suppression of $I_{\text{no}}$ by less permeant anions is not by a direct influence of actin cytoskeleton.

Basal activity of Cl\textsuperscript{−} channels in rat ventricular myocytes. In a symmetrical Cl\textsuperscript{−} gradient (Cl\textsubscript{in}\textsuperscript{−} = Cl\textsubscript{out}\textsuperscript{−} = 135 mM), a basal time-independent current was observed (Fig. 8A). The reversal potential of the basal current was $-1.1 ± 1.2$ mV ($n = 4$). This was close to the Cl\textsuperscript{−} equilibrium potential ($E_{\text{Cl}} = 0$ mV) predicted by the Nernst Equation. Replacement of 125 mM Cl\textsuperscript{−} with Asp\textsuperscript{−} shifted the reversal potential from $-1.1 ± 1.2$ to $+6.25 ± 3.8$ mV ($n = 4$), close to the new $E_{\text{Cl}} (+67$ mV), whereas Br\textsuperscript{−} substitution for Cl\textsuperscript{−} did not induce significant shift of the reversal potential ($-0.5 ± 1.3$ mV, $n = 4$). These results suggest that the basal current is carried by Cl\textsuperscript{−}. The anion permeability sequence of the channel, obtained from the shifts of the reversal potentials of the basal Cl\textsuperscript{−} current, was Cl\textsuperscript{−} ≈ Br\textsuperscript{−} ≫ Asp\textsuperscript{−} (Fig. 8B). These data indicate that there is a basal activity of Cl\textsuperscript{−} channels in rat cardiac ventricular myocytes under basal conditions.

DISCUSSION

The main findings in the present study are as follows: 1) The activity of $I_{\text{no}}$ in rat ventricular myocytes displays an anionic permeability-dependent property, i.e., $I_{\text{no}}$ is facilitated in permeant anion-rich extracellular solution, but it is suppressed by less permeant Cl\textsuperscript{−} substitutes. The effects of the anions on $I_{\text{no}}$ are the result of a shift of the steady-state inactivation curve. 2) Depolymerization of actin microfilaments with CytD, like less permeant anions, shifts the steady-state inactivation curve to a more negative potential.
The cardiac $I_{\text{to}}$ was initially thought to be carried by $\text{Cl}^-$ because it decreased in $\text{Cl}^-$-free solution (13, 15, 18). However, subsequent studies indicated that this current was primarily carried by $\text{K}^+$ (24). The effects of $\text{Cl}^-$ substitution on the $I_{\text{to}}$ are usually attributed to altered $\text{Ca}^{2+}$, since less permeant $\text{Cl}^-$ substitutes may reduce the free $\text{Ca}^{2+}$ concentration (23) and consequently inhibit a $\text{Ca}^{2+}$-activated $\text{Cl}^-$ current (47). However, in rat cardiac ventricular myocytes, the $I_{\text{to}}$, as a matter of fact, is a sole current carried by $\text{K}^+$ ($I_{\text{to}}$), which is independent of $\text{Ca}^{2+}$ and sensitive to blockade by 4-AP (3, 14, 22). Both biochemical and functional evidence shows that $\text{K}_v4.2$ and $\text{K}_v4.3$ contribute to the cardiac $I_{\text{to}}$ (10, 11, 35). Although rat cardiac $I_{\text{to}}$ is also inhibited by less permeant anions, Lefevre et al. (30) have demonstrated that the inhibitory effect of the anions is not significantly altered when $\text{Ca}^{2+}$ is removed and $\text{Ca}^{2+}$ is buffered with a high concentration of EGTA. In the present study, it is unlikely that the effect of less permeant anions was the result of changes in free $\text{Ca}^{2+}$ because $\text{Asp}^-$ and $\text{Glu}^-$, which only cause a slight decrease in free $\text{Ca}^{2+}$ (<2.3%, see MATERIALS AND METHODS), induce a more markedly inhibitory effect than $\text{Glc}^-$ does. The latter markedly decreased the free $\text{Ca}^{2+}$ by 68.3%. These unparallel effects of the less permeant anions on the free $\text{Ca}^{2+}$ and $I_{\text{to}}$ suggest that their action may involve a mechanism other than chelation of $\text{Ca}^{2+}$. It may be argued that a fraction of $I_{\text{to}}$ may be carried by $\text{Cl}^-$. If $\text{Cl}^-$ can pass through the $I_{\text{to}}$ channel, then the residual $I_{\text{to}}$ carried by $\text{Cl}^-$ should remain after removing $\text{K}^+$. However, when the myocytes were dialyzed with the pipette solution in which 130 mM $\text{K}^+$ was replaced by equimolar $\text{Cs}^+$, $I_{\text{to}}$ decreased progressively and disappeared within 4 min, although the concentrations of both $\text{Cl}^-$ and $\text{Cl}^-$ remained unchanged ($n = 4$, data not shown). This is similar to the observation reported by Lefevre et al. (30). Therefore, it is unlikely that $\text{Cl}^-$ is a carrier of $I_{\text{to}}$. In the present study, a KCl-agar bridge electrode was used to minimize the junction potential caused by alteration in $\text{Cl}^-$ concentration. If the KCl-agar bridge electrode failed to effectively reduce the liquid junction potentials, changes in liquid junction potentials should alter the activation of both $I_{\text{to}}$ and the sustained component of outward $\text{K}^+$ current. However, less permeant anions, like the specific $I_{\text{to}}$ inhibitor 4-AP, only inhibited $I_{\text{to}}$, whereas the sustained component of outward $\text{K}^+$ current was not significantly affected. Thus it is unlikely that inhibition of $I_{\text{to}}$ by less permeant anions is caused by changes in liquid junction potentials. The present data suggest that the effects of $\text{Cl}^-$ and its substitutes on cardiac $I_{\text{to}}$ involve an anion-related factor.

In cardiac myocytes, the physiological $\text{K}^+$ gradient is maintained by a combination of outward $\text{K}^+$ movement through $\text{K}^+$ channels and an inward movement via the $\text{Na}^+-\text{K}^+-\text{Cl}^-$ cotransporter (32) and the activity of $\text{Na}^+-\text{K}^+$-ATPase. Inhibition of $\text{Na}^+-\text{K}^+-\text{Cl}^-$cotransport may cause accumulation-depletion of $\text{K}^+$ in situ, i.e., an increase in the external $\text{K}^+$ ($K_e$) and a decrease in the $K_i^+$, thereby reducing the driving force for $K^+$. Thus the inhibition of $I_{\text{to}}$ by less permeant anions may be the result of an inhibition of $\text{Na}^+-\text{K}^+-\text{Cl}^-$ cotransport, as proposed by Lefevre et al. (30). However, in contrast to the less permeant anions, substitution of $\text{Cl}^-$ by some permeant anions, which should also impair $\text{Na}^+-\text{K}^+-\text{Cl}^-$cotransport, was found to facilitate $I_{\text{to}}$ rather than inhibit it. Moreover, the driving force for $\text{K}^+$ is not expected to be significantly altered in $\text{Cl}^-$ substitution conditions because the $K_i^+$ concentration is clamped at a constant level in the whole cell patch-clamp configuration, and the $K_i^+$ is maintained constant by continual perfusion. $\text{Na}^+$ is also an important

Fig. 8. Basal $\text{Cl}^-$ current in rat ventricular myocytes. Whole cell basal $\text{Cl}^-$ currents were elicited from a holding potential of $-40 \text{ mV}$ to test potentials ranging from $-100$ to $+100 \text{ mV}$ in $20-\text{mV}$ increments. $A$: representative whole cell currents recorded under a symmetrical $\text{Cl}^-$ gradient condition ($\text{Cl}^-=135 \text{ mM}$; $a$) and after replacement of 125 mM $\text{Cl}^-$ with equimolar $\text{Br}^-$ (b) and $\text{Asp}^-$ (c). Arrows indicate 0 current level. $B$: current ($I$)-voltage relationships of the currents in $A$.
factor for stimulating Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport and Na\(^{+}\)-K\(^{+}\)-ATPase; substitution of Na\(^{+}\) with an impermeant cation should eliminate Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport. However, removal of Na\(^{+}\) does not affect the inhibitory action of less permeant anions (Fig. 2). Furthermore, intracellular Na\(^{+}\) has also been found to have no effects on \(I_{\text{to}}\) (14). Inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase with its specific inhibitor ouabain (0.5 \(\mu\)M) does not markedly influence the action of the less permeant anion Asp\(^{-}\) (\(n = 5\), data not shown). Therefore, it seems unlikely that the inhibitory effect of less permeant anions on \(I_{\text{to}}\) is caused by either an impairment of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport or alteration of the activity of Na\(^{+}\)-K\(^{+}\)-ATPase.

Anion channels exist ubiquitously in cells, including rat ventricular myocytes (12, 26, 42), and display distinct anionic selectivity and permeability sequences (21). Studies have revealed that external anions may modulate the gating of some anion channels (38, 39). In the present study, a basal Cl\(^{-}\) channel activity was observed. The Cl\(^{-}\) channel had an anion permeability sequence of Cl\(^{-}\) > Br\(^{-}\) > Asp\(^{-}\), which matched the action of these anions on \(I_{\text{to}}\). Thus the present results could not rule out the possibility that the \(I_{\text{to}}\) channel may be modulated by anion channel(s) via a channel-channel interaction. Recent evidence reveals that the actin cytoskeleton is implicated in the regulation of a variety of ion channels, including voltage-gated K\(^{+}\) channels (17, 33, 34, 36) and anion channels (2, 6, 7, 37, 45). A previous study on epithelial anion channels has suggested that actin plays a role in channel-channel interaction (20). The present study found that disrupting the actin microfilaments with CytD produced an effect similar to that induced by less permeant anions. Confocal imaging revealed that CytD induced a disruption of actin microfilaments. In contrast, less permeant anion Asp\(^{-}\) did not affect the structure of the actin cytoskeleton. It is unlikely that less permeant anions affect \(I_{\text{to}}\) by directly acting on the actin cytoskeleton. These results suggest that the actin cytoskeleton may play a linkage role in the interaction between the anion channel and \(I_{\text{to}}\) channel in rat cardiac ventricular myocytes, direct evidence for the interaction between the Cl\(^{-}\) channel and \(I_{\text{to}}\) channel is lacking. Further work is required to test this hypothesis.

In many mammalian hearts, including humans, \(I_{\text{to}}\) is mainly responsible for the initial rapid phase of the action potential repolarization. Defects of the \(I_{\text{to}}\) channel may result in both abnormal electrical activities (28, 35) and morphological changes in the heart (5, 28). Therefore, investigation into the regulation of cardiac \(I_{\text{to}}\) is of clinical importance. The present study provides new insight into the regulation of cardiac \(I_{\text{to}}\).

ACKNOWLEDGMENTS

We thank Dr. G. Droogmans for the kind gift of the CaBuf program.

GRANTS

This research was supported by National Natural Science Foundation of China Grants 39870318 and 30270602 and the Sun Yat Sen Foundation Fund from the University of Hong Kong.

REFERENCES

1. Accili EA and DiFrancesco D. Inhibition of the hyperpolarization-activated current (\(I_{\text{h}}\)) of rabbit SA node myocytes by niflumic acid. Pfliigers Arch 431: 757–762, 1996.


