Chloride Current in Mammalian Cardiac Myocytes

**Novel Mechanism for Autonomic Regulation of Action Potential Duration and Resting Membrane Potential**

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**ABSTRACT** The properties of the autonomically regulated chloride current ($I_{Cl}$) were studied in isolated guinea pig ventricular myocytes. This current was elicited upon exposure to isoproterenol (ISO) and reversed upon concurrent exposure to acetylcholine (ACh). $I_{Cl}$ was time independent and exhibited outward rectification. The responses to ISO and ACh could be blocked by propranolol and atropine, respectively, and $I_{Cl}$ was also elicited by forskolin, 8-bromoadenosine 3',5'-cyclic monophosphate, and 3-isobutyl-1-methylxanthine, indicating that the current is regulated through a cAMP-dependent pathway. The reversal potential of the ISO-induced current followed the predicted chloride equilibrium potential, consistent with it being carried predominantly by Cl⁻. Activation of $I_{Cl}$ produced changes in the resting membrane potential and action potential duration, which were Cl⁻ gradient dependent. These results indicate that under physiological conditions $I_{Cl}$ may play an important role in regulating action potential duration and resting membrane potential in mammalian cardiac myocytes.

**INTRODUCTION**

The electrophysiological properties of muscle tissue have been a topic of interest for many decades. Early on it was found that skeletal muscle exhibits a large chloride (Cl⁻) conductance. At rest, the ratio of Cl⁻ permeability to K⁺ permeability is on the order of 2:1 (Hodgkin and Horowicz, 1959; Hutter and Noble, 1960). The resting permeability of cardiac muscle, on the other hand, is dominated by K⁺. However, evidence of a chloride conductance in cardiac tissue has been reported (Carmeliet, 1961; Hutter and Noble, 1961). Although the relative permeability of Cl⁻ to K⁺ at the resting membrane potential was considered to be minor, changes in action potential configuration and spontaneous activity were observed in Purkinje fibers that were exposed to Cl⁻-free solutions. From this it was concluded that a chloride conductance
conductance played an important role in action potential repolarization as well as diastolic depolarization.

Subsequent application of voltage-clamp techniques to the study of membrane currents in cardiac preparations led to the discovery of a transient outward current which was believed to play an important role in action potential repolarization (Deck et al., 1964; Deck and Trautwein, 1964). Furthermore, reduction of this current in Cl\(^{-}\)-free solutions suggested that it was conducted by Cl\(^{-}\) (Dudel et al., 1967). This transient outward current (\(I_{Ow}\)) was referred to as the positive dynamic current (Peper and Trautwein, 1968), the initial outward current (Reuter, 1968), or \(I_{qr}\) (McAllister et al., 1975). The properties of this proposed Cl\(^{-}\) current were studied extensively (Vitek and Trautwein, 1971; Fozzard and Hiraoka, 1973; Hiraoka and Hiraoka, 1973, 1975; Gibbons and Fozzard, 1975), and \(I_{qr}\) was included as a repolarizing current in early models of electrical activity in Purkinje fibers (McAllister et al., 1975). However, it was later shown by Kenyon and Gibbons (1977) that the apparent Cl\(^{-}\) dependence of \(I_{Ow}\) was due to changes in external Ca\(^{2+}\) activity caused by chelation of free Ca\(^{2+}\) by the Cl\(^{-}\) substitutes. This was supported by findings of a Ca\(^{2+}\) dependence of the transient outward current (Siegelbaum et al., 1977; Siegelbaum and Tsien, 1980), and the fact that \(I_{Ow}\) was much less sensitive to replacement of extracellular Cl\(^{-}\) when free Ca\(^{2+}\) was maintained at a constant level (Kenyon and Gibbons, 1979a). In addition, it was shown that \(I_{Ow}\) could be blocked by the potassium channel antagonists tetroethylammonium (TEA) and 4-aminopyridine (Kenyon and Gibbons, 1979a, b), suggesting that it was actually a K\(^{+}\) current. Subsequent studies have demonstrated that the transient outward current consists of Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent components (Coraboeuf and Carmeliet, 1982; Escande et al., 1987; Hiraoka and Kawano, 1989; Tseng and Hoffman, 1989), and that the Ca\(^{2+}\)-independent \(I_{Ow}\) is carried predominantly by K\(^{+}\) (Giles and Van Ginneken, 1985; Nakayama and Irisawa, 1985; Hiraoka and Kawano, 1989). Therefore, the existence of a Cl\(^{-}\) current in cardiac muscle appeared to be in doubt.

There have been reports of single Cl\(^{-}\)-conducting channels from cardiac sarcolemmal proteins that have been inserted into lipid bilayers (Coronado and Latorre, 1982, 1983; Hill et al., 1989). However, no corresponding macroscopic currents have been identified. A transient, tetrodotoxin (TTX)-sensitive current has been reported in rat ventricular myocytes that may possibly be carried by Cl\(^{-}\) (Pidoplichko and Verkhratsky, 1987), but this has yet to be confirmed. Recently we reported finding a background current that is elicited upon exposure to isoprotrenol (Harvey and Hume, 1989a). The reversal potential of this current varies with changes in intracellular Cl\(^{-}\) concentration, strongly suggesting that this is a Cl\(^{-}\) current. In the present report, we further investigated the ionic nature of this current as well as its mechanism of regulation, sensitivity to Cl\(^{-}\) channel antagonists, and role in regulating action potential duration and resting membrane potential.

**METHODS**

*Preparation of Myocytes*

The methods used to isolate guinea pig ventricular myocytes were a modification of those previously described (Hume and Uehara, 1985). Briefly, hearts were rapidly excised and coronary vessels were cleared of blood by retrograde perfusion of the aorta with a Krebs-Hense-
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Liet buffer solution (KHB) containing (in millimolar): 120 NaCl, 4.8 KCl, 1.5 CaCl₂, 2.2 MgSO₄, 1.2 NaH₂PO₄, 25 NaHCO₃, and 10 glucose, equilibrated with 95% O₂-5% CO₂ at 37°C. The heart was perfused at a constant pressure for 5 min with the normal KHB, then the solution was switched to nominally Ca²⁺-free KHB for 5 min. After this, collagenase (type I, Sigma Chemical Co., St. Louis, MO) was added to the Ca²⁺-free KHB (65 U/ml) and the solution was recirculated while digestion continued for 45 min. The ventricles were then removed, cut into small pieces (2 × 2 mm), placed in a flask containing collagenase (175 U/ml) in Ca²⁺-containing KHB, and incubated for an additional 30 min. After this the pieces of ventricle were placed in normal, collagenase-free KHB, and single cells were obtained by gentle trituration.

Voltage-Clamp Technique

Whole-cell membrane currents were measured using the patch-clamp technique in the whole-cell configuration described by Hamill et al. (1981). Suction pipettes were made using borosilicate glass capillary tubing (1.2 mm i.d.; Richland Glass, Vineland, NJ). Pipettes filled with intracellular solution had tip resistances ranging between 0.5 and 2 MΩ. All data were compensated for liquid junction potential artifacts. Current recordings were obtained using a List EP7 voltage-clamp amplifier (Medical Systems Corp., Greenvale, NY). Currents were filtered at 3 kHz and recorded digitally using an IBM AT computer and pCLAMP software (Axon Instruments, Inc., Burlingame, CA). All results are reported as the mean ± SD.

Experimental Solutions

The control external solution was a modified Tyrode's solution with the following composition (in millimolar): 140 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 5.5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10 glucose. The pH was adjusted to 7.4 with NaOH. In experiments in which extracellular Na⁺ was removed, NaCl was replaced with equimolar concentrations of either tetramethylammonium (TMA) chloride, N-methyl-D-glucamine HCl, or choline chloride. The external solution used to block K⁺ current was prepared by either adding 400 μM BaCl₂ to the control external solution or by replacing external KCl with equimolar concentrations of CsCl. The external concentration of Cl⁻ was altered by replacing NaCl with an equimolar concentration of Na-aspartate. Calcium current was blocked by adding either 1 μM nisoldipine or 100 μM CdCl₂ to the external solution, except where noted.

The control intracellular solution contained (in millimolar): 130 K-aspartate (or glutamate), 10 KCl, 10 NaCl, 1 MgCl₂, 1 K₇ATP, 5 ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 5 HEPES. The pH was adjusted to 7.2 with KOH. The intracellular solution used to block K⁺ currents contained (in millimolar): 110 Cs-aspartate, 20 TEA chloride, 10 CaCl₂, 10 NaCl, 1 MgCl₂, 1 K₇ATP, 5 EGTA, and 5 HEPES. In some experiments, the intracellular solution contained 5 mM MgATP. The intracellular Cl⁻ concentration was varied by replacing K-aspartate (or glutamate) with equimolar concentrations of KCl.

RESULTS

The resting conductance of guinea pig ventricular myocytes is dominated by the inward-rectifying K⁺ current (Iₖᵡ), but because of its strongly rectifying nature, little if any outward current associated with Iₖᵢ can be observed at membrane potentials more positive than ~20 mV (see Fig. 2 B). The delayed rectifier K⁺ current (Iₖᵢ), on the other hand, is activated at these potentials (Matsuura et al., 1987; Harvey and Hume, 1989b). However, activation of Iₖᵢ is not evident during short voltage-clamp
pulses because this current exhibits a lag in onset (Matsuura et al., 1987; Hume et al., 1989) and activation is slowed when experiments are carried out at room temperature (see Fig. 9). Furthermore, guinea pig ventricular myocytes exhibit little, if any, transient outward \( K^+ \) current \( (I_{to}) \). Therefore, changes in background current were easily monitored by applying 100-ms voltage-clamp steps to positive membrane potentials, under conditions where \( Na^+ \) current was inactivated by using a depolarized holding potential (between -30 and -40 mV) and \( Ca^{2+} \) current was blocked by adding either nisoldipine (1 \( \mu \)M) or \( Cd^{2+} \) (100 \( \mu \)M) to the extracellular solutions.

**Isoproterenol-induced Currents**

An example of the effect of isoproterenol (ISO) is shown in Fig. 1. The control current trace shows that there is little outward current evident at +52 mV. However, after application of 1 \( \mu \)M ISO, there was an increase in the outward current elicited during depolarization. The ISO-induced current appeared to be time independent, and it was reversed upon concurrent exposure to 10 \( \mu \)M acetylcholine (ACh). Fig. 1 B illustrates the time course of the response to ISO and ACh, as well as the reversibility of the reaction to both compounds.

Rectification of \( I_{K1} \) is clearly illustrated by the family of currents shown in Fig. 2 A, a, which were recorded under control conditions. The presence of the negative slope conductance region between -60 and -20 mV is more clearly seen in the current-voltage relationship shown in Fig. 2 B. However, after application of ISO, there was a change in the background current which was most obvious as an increase of the outward current at potentials positive to -40 mV (Fig. 2 A, b). The current-voltage relationship (Fig. 2 B) also indicated that there was an increase, although smaller, of the inward current at more negative potentials. Furthermore, ACh reversed the response to ISO at all potentials (Fig. 2 A, c; Fig. 2 B).

To obtain a better impression of the current that was being modulated by ISO and ACh, the ISO-induced current was isolated by subtracting membrane currents recorded under control conditions from those obtained after exposure to ISO. A family of these difference currents is shown in Fig. 3 A. The ISO-induced current, as expected from looking at the raw currents, did not appear to exhibit any time dependence. However, as indicated in Fig. 2 B, the ISO-induced current did exhibit an outwardly rectifying voltage dependence. This is illustrated in the current-voltage relationship of the difference current (Fig. 3 B). Under these conditions the current reversed at -44.2 ± 9.2 mV (n = 5).

**Regulatory Mechanism**

The responses to ISO and ACh were further characterized by identifying the receptors involved. Fig. 4 A illustrates the effect of atropine and propranolol on the responses to ACh and ISO, respectively. Changes in the background current were monitored by applying 100-ms voltage-clamp pulses to +52 mV, from a holding potential of -38 mV at a frequency of 0.05 Hz. The current elicited during the test pulse is plotted as a function of time. As shown previously, exposure to ISO (1 \( \mu \)M) elicited an outward current, and ACh reversed this response. Concurrent exposure to atropine (1 \( \mu \)M) blocked the ACh effect, resulting in the return of the ISO-
induced current, and the response to ISO was blocked by propranolol (10 μM). Thus it appears that the current elicited by ISO and antagonized by ACh is regulated via β-adrenergic and muscarinic receptors.

The fact that two other currents in cardiac myocytes, $I_{Ca}$ and $I_K$, are regulated by β-adrenergic and muscarinic agonists in an antagonistic fashion (Hescheler et al., 1986; Harvey and Hume, 1989b), suggested that the ISO-induced background current may be controlled through a similar pathway. Both $I_{Ca}$ and $I_K$ are enhanced...
after β-adrenergic receptor stimulation, through a pathway that involves activation of adenylate cyclase and the subsequent production of the second messenger, adenosine 3',5'-cyclic monophosphate (cAMP) (Walsh et al., 1989). To determine whether cAMP could be involved in regulating the background current elicited by

Figure 2. Voltage dependence of the ISO- and ACh-induced changes in membrane current. (A) Families of current elicited by 100-ms voltage-clamp pulses to membrane potentials between -98 and +52 mV in 10-mV steps from a holding potential of -38 mV under unstimulated conditions (a), after exposure to 1 µM ISO (b), and after exposure to ISO + 10 µM ACh (c). (B) Current-voltage-relationship of currents shown in A. Control intra- and extracellular solutions (E_int, -49 mV). Ca^{2+} current was blocked using 1 µM nisoldipine.

ISO, cells were exposed to forskolin (FSK), a compound that directly activates adenylate cyclase, resulting in the production of cAMP (Seamon and Daly, 1986). FSK evoked a time-independent current which resembled that elicited by ISO. The FSK-induced current exhibited a reversal potential of $-44.3 \pm 4.7$ mV ($n = 3$) and an
outwardly rectifying voltage dependence (Fig. 4 B). Furthermore, the FSK-induced current was reversed by concurrent exposure to ACh. These results imply that FSK activates the same current as ISO, suggesting that the ISO-induced current is indeed regulated by cAMP. Regulation by cAMP is further supported by the fact that the time-independent background current was also elicited by the exposure of cells to the membrane-permeable derivative of cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate, as well as the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine. These results are consistent with those of Bahinski et al. (1989).

**FIGURE 3.** ISO-induced difference current. (A) ISO-induced difference current obtained by subtraction of digitized current traces recorded under unstimulated (CTL) conditions from current traces recorded after exposure to 1 µM ISO, using the voltage-clamp protocol shown below. (B) Current-voltage relationship of ISO-induced difference currents obtained from the same cell used in A. Solid line through data points was fit by eye. Control intra- and extracellular solutions (E<sub>IC</sub>, -49 mV). Ca<sup>2+</sup> current was blocked using 1 µM nisoldipine.

**Ionic Nature**

The observation that β-adrenergic stimulation elicited a time-independent background current was quite unexpected. One possible explanation was that ISO was regulating a known current. However, the fact that Na<sup>+</sup> current was inactivated by the holding potential and Ca<sup>2+</sup> current was blocked by either nisoldipine or Cd<sup>2+</sup> placed doubt on the involvement of these two types of channels.

Because the reversal potential of the ISO-induced current was quite negative, K<sup>+</sup>...
seemed a more likely candidate for the charge carrier. The fact that the ISO-induced current was time independent suggested that the response was not an effect on a time-dependent K⁺ current such as I_K. However, guinea pig ventricular myocytes are known to possess ATP-sensitive K⁺ channels. When activated, these channels produce an apparent time-independent macroscopic current upon depolarization (Noma and Shibasaki, 1985). However, the ISO-induced background current was not affected by subsequent exposure to 10 μM glibenclamide, an effective blocker of ATP-sensitive K⁺ channels (Escande et al., 1988; Sanguinetti et al., 1988). This is consistent with the fact that the current exhibited outward rectification, whereas the ATP-sensitive K⁺ current would be expected to exhibit inward rectification (Noma and Shibasaki, 1985) under these conditions.

Although the ISO-induced current did not appear to be conducted through ATP-sensitive K⁺ channels, it was still possible that this current represents a modification of the rectifying behavior of I_K. To ascertain whether the current evoked by ISO was a K⁺ current, experiments were carried out using cells that were dialyzed with a pipette solution containing Cs⁺ and TEA. In these experiments, the membrane potential was held at −38 mV and the background current was measured after 100-ms test pulses to potentials from −128 to +42 mV. Current traces recorded before

**Figure 4.** Regulatory pathway of ISO- and ACh-induced changes in background current. (A) Time course of changes in background current elicited during a 100-ms voltage-clamp pulse to +52 mV from a holding potential of −38 mV applied at a frequency of 0.05 Hz. The cell was exposed to 1 μM ISO, 10 μM ACh, 1 μM atropine (ATROP), and 10 μM propranolol (PROP) at the times indicated. (B) Current-voltage relationship of the time-independent difference current obtained by subtraction of digitized current traces recorded under unstimulated conditions from current traces recorded after exposure to 1 μM FSK. Solid line through data points was fit by eye. Control intracellular and extracellular solutions (E_out, −49 mV). Ca²⁺ current was blocked using 1 μM nisoldipine.
and after exposure to ISO (1 μM) are illustrated in Fig. 5 A. It is clear that the ISO-induced current was still evocable, and its voltage dependence was not affected (Fig. 5 B), suggesting that the current was not carried by K⁺. Similar results were obtained when K⁺ currents were blocked using extracellular Ba²⁺.

Another possible explanation for the ISO-induced background current could be that the Na,K pump was being stimulated. It has been suggested that the activity of this electrogenic exchanger is increased by β-adrenergic stimulation (Desilets and...
However, 100 μM ouabain did not affect the background current elicited by ISO. The probability that activation of the Na,K pump is involved in the response to ISO was further placed in doubt by dialyzing cells with a Na-free internal solution and bathing them in Na-free external solution. Under these conditions any exchange mechanism involving Na⁺ should have been completely inhibited. However, exposure to ISO still elicited the background current (Fig. 6 A). The current elicited in the absence of Na⁺ was time independent, exhibited outward rectification, and reversed at $-43.3 \pm 10.4$ mV ($n = 8$). Because the reversal poten-

![Figure 6](image-url)

Figure 6. Sodium dependence of the ISO-induced current. (A) ISO-induced difference current obtained by subtraction of digitized current traces recorded under unstimulated conditions from current traces recorded after exposure to 1 μM ISO. Solid line through data points was fit by eye. Intracellular solution contained no Na⁺ (replaced by K⁺); extracellular solution contained no Na⁺ (replaced by N-methyl-D-glucamine) ($E_{\text{in}}$, $-49$ mV). Ca²⁺ current was blocked using 1 μM nisoldipine. (B) Time course of changes in background current elicited during a 100-ms voltage-clamp pulse to $+50$ mV from a holding potential of $-30$ mV applied at a frequency of 0.05 Hz. The cell was exposed to 1 μM ISO. After the appearance of the ISO-induced background current, the cell was exposed to an extracellular solution containing 1 μM ISO without Na⁺ (replaced by TMA). Subsequently, 10 μM ACh was added. Intracellular solution contained 10 mM Na⁺; control extracellular solution, except as noted ($E_{\text{in}}$, $-49$ mV). Ca²⁺ current was blocked using 1 μM nisoldipine.

...tial, under these conditions, was not significantly different than that determined in the presence of Na⁺ ($-44.2 \pm 9.2$ mV, $n = 5$; $P > 0.4$), it is clear that the ISO-induced current was not carried by this monovalent cation. However, when the background current was first elicited by ISO, and then extracellular Na⁺ was removed, the magnitude of the current was reduced. This phenomenon is illustrated in Fig. 6 B. Changes in the background current were monitored by applying voltage-clamp pulses to $+50$ mV from a holding potential of $-30$ mV. After eliciting the...
background current with 1 μM ISO, the control extracellular solution was replaced with a Na-free solution which also contained ISO. This resulted in a decrease in the size of the ISO-induced current. The magnitude of the reduction upon removal of Na⁺ varied, but the ISO-induced current was never completely eliminated. Similar results were obtained when extracellular Na⁺ was replaced with either N-methyl-D-glucamine, choline, or TMA. In the experiment shown in Fig. 6 B, the cell was dialyzed with an intracellular solution containing 10 mM Na⁺. The effect of removing extracellular Na⁺ appeared to be even more pronounced when cells were dialyzed with a Na⁺-free intracellular solution. These results indicate that while the ISO-induced current is not carried by Na⁺, it is Na sensitive.

The data presented thus far suggest that the time-independent current elicited by β-adrenergic stimulation was not a modification of a previously described current nor was it clear that any cation contributed to the current. Another alternative was that this current might be conducted by an anion, specifically Cl⁻. In the initial experiments (see Figs. 1–4) the intracellular Cl⁻ concentration was 22 mM and the extracellular Cl⁻ concentration was 151.4 mM. The predicted Cl⁻ equilibrium potential (E_G) under these conditions would be −49 mV, which is very close the reversal potential of the ISO- and FSK-induced current (−44 mV). Furthermore, in the experiments shown in Fig. 5, the intracellular Cl⁻ concentration was 42 mM, while the extracellular Cl⁻ concentration remained the same. Under these conditions, the reversal potential of the ISO difference current shifted to −23.4 ± 7.6 mV (n = 11). This corresponds to the predicted E_G of −33 mV. Here again, the ISO-induced current exhibited pronounced outward rectification. These results were consistent with the idea that the ISO-induced background current might be carried by Cl⁻.

The Cl⁻-dependent nature of the ISO-induced current was further tested by increasing the intracellular Cl⁻ concentration to 152 mM (E_G, 0 mV). Fig. 7 A shows membrane currents elicited by voltage-clamp pulses to +50 mV, from a holding potential of −30 mV, before and after exposure to ISO. As before, the outward current during the test pulse was increased. However, under these conditions, exposure to ISO had a much larger effect on the holding current at −30 mV; in fact, before ISO the holding current was outward and after ISO the holding current became inward. The effect of ISO on membrane currents between −80 and +50 mV is illustrated in the current-voltage relationship of the total membrane current (Fig. 7 B). In this example, the total background current exhibited three reversal potentials in the presence of ISO, which was caused by the inward shift of the N-shaped current-voltage relationship of I_K1 at negative membrane potentials. The total background current in some cells reversed at only one potential. This was usually within the more negative of the two positive slope conductance regions of the current-voltage relationship. In cells dialyzed with 152 mM Cl⁻, the ISO-induced current reversed at 0.7 ± 3.4 mV (n = 16). Fig. 7 C illustrates the current-voltage relationship of the ISO-induced difference current. Unlike experiments in which cells were dialyzed with lower intracellular Cl⁻ concentrations, the voltage dependence of the difference current under these conditions was linear.

The Cl⁻-dependent nature of the ISO-induced current was also apparent when E_G was altered by adjusting the extracellular Cl⁻ concentration. When Cl⁻ was
maintained at 152 mM and Cl\textsuperscript{-} was lowered to 75 mM (E\textsubscript{Cl\textsuperscript{-}} +18 mV), the ISO-induced current reversed at +12.3 ± 2.5 mV (n = 3). Fig. 8A illustrates the relationship between the experimentally determined reversal potential of the ISO-induced current and the predicted Cl\textsuperscript{-} equilibrium potential. This relationship (slope, 0.81) closely followed the unity relationship expected for a pure Cl\textsuperscript{-} current.

![Figure 7. Effect of ISO on the background current in cells dialyzed with an intracellular solution containing 152 mM Cl\textsuperscript{-}. (A) Membrane current elicited during a 100-ms voltage-clamp step to +50 mV from a holding potential of -30 mV, before (open circles) and after (closed circles) exposure to 1 μM ISO. (B) Current-voltage relationship of total membrane current which was measured during 100-ms voltage-clamp steps to membrane potentials between -80 and +50 mV from a holding potential of -30 mV, before (open circles) and after (closed circles) exposure to 1 μM ISO. Same cell as in A. Intracellular solution contained 152 mM Cl\textsuperscript{-} (replacing aspartate); the control extracellular solution was used (E\textsubscript{Cl\textsuperscript{-}}, 0 mV). Ca\textsuperscript{2+} current was blocked using 1 μM nisoldipine. (C) Current-voltage relationship of ISO-induced difference current obtained by subtraction of digitized current traces recorded under unstimulated conditions from current traces recorded after exposure to 1 μM ISO. Solid line through data points was fit by eye. Intracellular solution contained 152 mM Cl\textsuperscript{-}; extracellular solution contained 400 μM Ba\textsuperscript{2+} (E\textsubscript{Cl\textsuperscript{-}}, 0 mV). Ca\textsuperscript{2+} current was blocked using 1 μM nisoldipine.

Using only those experiments in which intracellular Cl\textsuperscript{-} was varied while extracellular Cl\textsuperscript{-} was kept at ~152 mM (i.e., excluding the data point for E\textsubscript{Cl\textsuperscript{-}} = +18 mV in Fig. 8A), the Nernst slope was 53 mV, close to the value of 59 mV expected for a pure Cl\textsuperscript{-} current under these experimental conditions. Therefore, we refer to the
ISO-induced current as $I_{Cl}$. The fact that the best correlation of the measured reversal potential and the predicted $E_{Cl}$ was under conditions where $Cl^-$ was the only anion on either side of the membrane ($E_{Cl}$, 0 mV) suggests that deviation from the predicted relationship at the other points may be due to some finite permeability of the channels to aspartate and glutamate.

**FIGURE 8.** (A) Chloride gradient dependence of the ISO-induced current. Relationship between the predicted $Cl^-$ equilibrium potential ($E_{Cl}$) and the measured reversal potential ($E_{rev}$) of the ISO-induced current. Each point is the mean ± SD of the number of experiments indicated. The solid line is a linear regression fit to the data points (slope, 0.81). The broken line is the predicted unity relationship (slope, 1) for a pure $Cl^-$ current. (B) Effect of the $Cl^-$ channel antagonist 9-AC on the ISO-induced current. The current-voltage relationship of the ISO-induced difference current was obtained by subtraction of digitized current traces recorded under unstimulated conditions from current traces recorded after exposure to 1 μM ISO, and the current-voltage relationship of the ISO-induced difference current was obtained after subsequent exposure to ISO + 100 μM 9-AC. Solid lines through data points were fit by eye. Intracellular solution contained 152 mM $Cl^-$; extracellular solution contained 400 μM $Ba^{2+}$ ($E_{Cl}$, 0 mV). $Ca^{2+}$ current was blocked using 1 μM nisoldipine.

**Chloride Channel Blockers**

Chloride currents have been identified in many different preparations, and several chloride conductance blockers have been identified. Although no one compound has been discovered that has a high affinity and is effective in all tissues, carboxylic...
Acid derivatives have been shown to reduce the chloride conductance in several skeletal muscle and epithelial preparations (Palade and Barchi, 1977; Wangemann et al., 1986; Gogelein, 1988). Therefore, the ISO-induced current was further characterized by examining the effect of one of these compounds, 9-anthracene carboxylic acid (9-AC). After eliciting the ISO-induced current, cells were exposed to 100 μM 9-AC extracellularly. The current was blocked by 9-AC, but the effect was very slow, suggesting that the compound may act at an intracellular site. Furthermore, the current was blocked in a voltage-independent manner (Fig. 8 B), with the slope conductance being reduced to 41.0 ± 23.7% of control (n = 4). This finding further supports the conclusion that the ISO-induced current was a Cl⁻ current.

Concentration Dependence of the ISO Response

Changes in the background current were observed when cells were exposed to concentrations of ISO as low as 10 pM. However, the appearance of ICl at this low concentration was transient. Concentrations of ISO >10 nM elicited a current which maintained a constant level as long as ISO was present. Concentrations >10 nM also appeared to elicit a maximal response. This was determined by activating ICl with various concentrations of ISO using cells dialyzed with 152 mM Cl⁻ (each cell was exposed to only one concentration). The slope conductance of the difference current was then calculated by fitting the current-voltage relationship between -50 and +50 mV to a straight line using a least-squares fitting routine. The conductance was then normalized to cell capacitance. The current density elicited by 10 nM ISO was 21.5 ± 10.4 S/F (n = 6). This was not significantly different (P > 0.5) from the current density observed in the presence of 1 μM ISO, which was 19.1 ± 7.4 S/F (n = 10).

Action Potentials and the Role of ICl

The intracellular Cl⁻ concentration measured in cardiac tissue varies between 10 and 24 mM (Vaughan-Jones, 1979; Fong and Hinke, 1980; Spitzer and Walker, 1980; Baumgarten and Fozzard, 1981; Desilets and Baumgarten, 1986b). Therefore, the characteristics of the current found using an internal solution containing 22 mM Cl⁻ may be similar to what is found in situ. To determine whether ICl might play an important role in controlling the action potential under these conditions, current-clamp experiments were performed using cells in which ICl had been elicited. These experiments were performed under conditions that would minimize action potential changes due to ISO-induced alterations of currents other than ICl. The Ca²⁺ current was blocked using 1 μM nisoldipine, and changes in IK were minimized by performing experiments at room temperature (Harvey and Hume, 1989b; Walsh et al., 1989). Fig. 9 illustrates the temperature-dependent response of IK. When experiments were performed at 37°C, not only was ICl elicited, but IK was also enhanced as indicated by the increase in magnitude of the time-dependent current during the test pulse and the increase in magnitude of the tail current observed on return to the holding potential (Fig. 9 A). However, when experiments were performed at 22°C, ICl could still be elicited, but there was no apparent change in the size of IK (Fig. 9 B).
FIGURE 9. Temperature dependence of the effect of ISO on the delayed rectifier K⁺ current (I_K), not the Cl⁻ current (I_Cl). (A) Current elicited during a 5-s voltage-clamp step to +50 mV from a holding potential of −30 mV under unstimulated conditions (CTL) and after exposure to 1 μM ISO, while the cell was maintained at 37°C. ISO increased the magnitude of I_K and elicited I_Cl. (B) Current was elicited using the same protocol as in A, while the cell was maintained at 22°C. ISO did not affect I_K, but I_Cl was still elicited. Control intra- and extracellular solutions (E_int, −49 mV). Ca²⁺ current was blocked using 1 μM nisoldipine.

Before recording action potentials (Fig. 10 A), membrane current was monitored (Fig. 10 B) using 5-s depolarizing voltage-clamp pulses to verify that rundown of I_K had reached a steady-state before recording action potentials and to verify that there were no ISO-induced changes in I_K. After exposure to ISO, I_Cl was evident as a

FIGURE 10. Action potentials and membrane current obtained from a cell exposed to ISO and ACh. (A) Action potentials evoked by intracellular current injection at a frequency of 0.25 Hz under unstimulated conditions (1), after exposure to 1 μM ISO (2), and after exposure to ISO + 10 μM ACh (3). (B) Membrane current recorded from the cell used to obtain the data in A; currents were recorded after each intervention, but before recording action potentials. Numbered traces correspond to legend in A. Currents were elicited by 5-s voltage-clamp steps to +42 mV from a holding potential of −38 mV. Control intra- and extracellular solutions (E_int, −49 mV). Ca²⁺ current was blocked using 1 μM nisoldipine.
parallel shift of what remained of $I_K$. Associated with the appearance of $I_{Cl}$ was a pronounced decrease in action potential duration as well as a slight depolarization of the resting membrane potential. Furthermore, addition of 10 $\mu$M ACh reversed all of the ISO-induced changes.

Even though the Ca$^{2+}$ current was effectively eliminated in the voltage-clamp experiments, it is possible that $I_{Ca}$ was not completely blocked during the action potential. Dihydropyridines such as nisoldipine exhibit a voltage-dependent block such that some of the current block observed when a holding potential of $-30$ mV was used during the voltage-clamp experiments may have been relieved at the resting membrane potential during current-clamp experiments. However, even if there
were some $\text{Ca}^{2+}$ current available during these action potentials, the depolarizing effect associated with any increase of $I_{\text{Ca}}$ was certainly overcome by the repolarizing effect of $I_{\text{Cl}}$. The control action potential duration (at 90% repolarization) in these experiments was $280.5 \pm 28.4$ ms ($n = 6$), and after exposure to ISO, the action potential was shortened to $156.5 \pm 47.0$ ms.

The assumption that the $\text{Ca}^{2+}$ current was blocked in the previous experiments is supported by the observation of the short action potential duration. In experiments performed without a $\text{Ca}^{2+}$ channel antagonist, the action potential duration was much longer ($611.5 \pm 86.8$ ms, $n = 6$). Furthermore, when $I_{\text{Ca}}$ was not blocked, the time course of changes in action potential duration was quite interesting (Fig. 11, A and B). After exposure to ISO there was an initial lengthening of the action potential duration in five of the six experiments. This was followed by a shortening of the action potential duration to $506.8 \pm 60.6$ ms. This represents a decrease of 17.1%. The shortening of the action potential was accompanied by a slight membrane depolarization, which was presumably due to the activation of $I_{\text{Cl}}$. In experiments in which $E_{\text{Cl}}$ was $-49$ mV, ISO depolarized the resting membrane potential by $2.0 \pm 1.3$ mV ($n = 12$).

One interpretation of this phenomenon is that the initial lengthening of the action potential was due to an increase of $I_{\text{Ca}}$, and the depolarization of the resting membrane potential and shortening of the action potential duration was due to activation of $I_{\text{Cl}}$. If the $\text{Cl}^{-}$ current is responsible for these changes in action potential duration and resting membrane potential, it should be possible to alter the response of these parameters to ISO treatment by changing the $\text{Cl}^{-}$ gradient. This hypothesis was tested using cells dialyzed with an internal solution containing 152 mM $\text{Cl}^{-}$ ($E_{\text{Cl}}$, 0 mV). Under these conditions 1 $\mu$M ISO lengthened the action potential duration (Fig. 11 C) by an average of 38.5% ($n = 8$). An example of the onset of this effect is shown in Fig. 11 C. Furthermore, in these same cells, ISO depolarized the resting membrane potential by $5.2 \pm 4.9$ mV, and in two of these an additional transient depolarization was observed, which was accompanied by spontaneous action potentials (Fig. 11 D). These ISO-induced changes are consistent with activation of a chloride conductance under conditions where $E_{\text{Cl}}$ is shifted in a depolarized direction. The more pronounced change in resting potential could be explained by the larger driving force for $\text{Cl}^{-}$ at the resting potential, and the lengthening of the action potential could be explained by the reduced driving force for $\text{Cl}^{-}$ at plateau potentials, allowing ISO-induced changes in $I_{\text{Ca}}$ to dominate changes in action potential duration.

**DISCUSSION**

**Identification of the ISO-induced Current**

It would seem rather unlikely that after many years of electrophysiological study of cardiac tissue, a new current of any physiological significance would be discovered. For this reason it is important to be certain that the current we have described can be attributed to a property of the membrane and not an artifact of the technique. For example, it would be easy to mistake the increase in background current seen upon exposure to ISO as a leak current associated with a change in seal resistance
between the electrode and the cell membrane. However, observation of the current upon exposure to ISO and consistent reversal of the ISO-induced changes by ACh was too coincidental to simply attribute to changes in the seal resistance.

Perhaps the most convincing reason why this current has not been reported previously is because changes in or induction of a time-independent current are not as obvious when one is studying the effects of β-adrenergic agonists on transient currents such as $I_{Ca}$ or $I_{w}$. For this reason it may be important to reassess previous descriptions of β-adrenergic stimulation on these transient currents. Concurrent activation of the background current would not affect the magnitude of any time-dependent component but would add or subtract a time-independent component which could be mistaken as an element of $I_{Ca}$ or $I_{w}$. In isolated canine Purkinje cells β-adrenergic stimulation has been reported to increase a time-independent component of $I_{w}$ (Nakayama and Fozzard, 1988; Nakayama et al., 1989), which may represent activation of a time-independent $Cl^-$ current similar to that described in our study. In rabbit ventricular myocytes, β-adrenergic stimulation induces a $Cl^-$ current, without significantly altering $I_{w}$ (Harvey and Hume, 1989c).

The ionic nature of the ISO-induced current was also quite unexpected. The $Cl^-$ gradient dependence of the reversal potential of the ISO-induced current is convincing evidence that $Cl^-$ is the principal charge carrier (see Fig. 8 A). The fact that the reversal potential follows the $Cl^-$ equilibrium potential is also consistent with the idea that the current is being conducted via an ion channel rather than an electrogenic exchange mechanism. If the latter were the case, the reversal potential would be expected to reflect the equilibrium potential of any other ion(s) involved in the exchange process.

It seems ironic that a $Cl^-$ current may play such an important role in repolarization of the action potential considering the fact $Cl^-$ was once believed to be the charge carrier of the transient outward current. However, it is possible that there may still be some component of the time-dependent outward current that is conducted by $Cl^-$. The ionic nature of the $Ca^{2+}$-dependent component of the transient outward current has yet to be verified, as has the $Ca^{2+}$ dependence of $I_{Ca}$. Furthermore, Pidoplichko and Verkhratsky (1986) have observed a transient current in rat ventricular myocytes that may be carried by $Cl^-$, and this current also exhibits a Na$^+$ dependence.

Resting Membrane Potential and $I_{Cl}$

There have been variable reports of β-adrenergic–induced alterations of the resting membrane potential in ventricular tissue. These include hyperpolarizations attributed to activation of the Na$^+$, K$^+$ pump (Desilets and Baumgarten, 1986a), and depolarizations attributed to activation of a Na$^+$ conductance (Egan et al., 1987, 1988). We have shown that the Na$^+$, K$^+$ pump is unlikely to represent a significant component of ISO-induced current in our experiments, and while the ISO-induced current we have described does exhibit a Na sensitivity, it is clearly a $Cl^-$ current. Therefore, ISO-induced depolarizations of the membrane potential appear to reflect an important aspect of the significance of $I_{Cl}$.

Under more physiological conditions the $Cl^-$ gradient is such that the equilibrium potential for $Cl^-$ would be expected to be between $-48$ and $-70$ mV. This is based
on measured intracellular Cl⁻ activities between 10 and 24 mM and the assumption that extracellular Cl⁻ is 152 mM. Under these conditions, the resting membrane conductance is dominated by I_K1. Therefore, activation of a current that exhibits outward rectification and a reversal potential only slightly more positive than E_K would be expected to have only a small effect on the resting membrane potential. This is consistent with the results of our experiments in which cells were dialyzed with an internal solution containing 22 mM Cl⁻. The predicted E_A was −49 mV, and the effect of ISO on the resting potential was a small, yet consistent, depolarization.

When cells were dialyzed with a higher Cl⁻ concentration (152 mM), a much more pronounced effect was observed. Exposure to ISO under these conditions was associated with a much larger depolarization and spontaneous activity. This is consistent with the observations of Egan et al. (1987, 1988). They reported that β-adrenergic stimulation produced large depolarizations of the resting membrane potential of isolated guinea pig ventricular myocytes. However, their experiments were performed using a technique that involved the use of electrodes containing high (molar) concentrations of Cl⁻. An intracellular loading with Cl⁻ would be expected and could explain the large depolarization caused by β-adrenergic stimulation. In addition, they observed spontaneous action potentials associated with ISO-induced depolarization, something we found only when cells were dialyzed with a high intracellular Cl⁻ concentration. The conclusion of Egan et al. (1987, 1988) was that the current responsible for the depolarization was carried predominantly by Na⁺, because removal of extracellular Na⁺ attenuated the response. We have confirmed their observation of a Na⁺ sensitivity of the current, but it is quite clear that the current is not carried by Na⁺ because we were able to observe it in the absence of intra- and extracellular Na⁺ (see Fig. 6 A), and because the reversal potential was not altered. Furthermore, the Cl⁻ gradient dependence of the reversal potential suggests that it is predominantly a Cl⁻ current.

The resting membrane potential we measured when the predicted E_A was 0 mV also depended upon the approach used. If the membrane potential was clamped at a depolarized level (i.e., −30 to −40 mV) and if after activation of I_C, the resting membrane potential was measured by switching the voltage-clamp amplifier to current-clamp mode, a much larger depolarization was observed (Harvey and Hume, 1989a). However, when the membrane potential was monitored continuously while exposing the cell to ISO, a depolarization was recorded which did not appear to be as large as that noted when switching from voltage-clamp mode. This can be explained by the fact that in cells dialyzed with 152 mM Cl⁻, depending on the size of I_C elicited, the background current-voltage relationship can exhibit three reversal potentials (see Fig. 7 B). While in voltage-clamp mode, holding at a depolarized membrane potential, current was being injected, so that upon switching to current-clamp mode the membrane potential would stabilize at the more positive level. However, activation of the chloride conductance without injection of current (while in current-clamp) resulted in a stabilization of the resting membrane potential at a less positive level. It would be interesting to speculate whether this phenomenon might contribute to the two levels of resting potential reported in Purkinje fibers (Wiggins and Cranefield, 1976; Gadsby and Cranefield, 1977; Carmeliet, 1982).
Residual autonomic tone could conceivably have maintained the activation of the chloride conductance in these multicellular preparations.

The role that $I_C$ might play in regulating the cardiac resting potential has only been examined in ventricular myocytes. It is possible that $I_C$ may have a more profound effect on the resting potential in other areas of the heart. In ventricular myocytes, the resting conductance is quite high due to the high density of inward rectifying K+ ($I_{K1}$) channels (Sakmann and Trube, 1984; Hume and Uehara, 1985). In atrial cells (Hume and Uehara, 1985) and pacemaker cells (Noma et al., 1984), the resting conductance is quite low compared with that of ventricular cells, due to the lower incidence of $I_{K1}$ channels in these cells. Therefore, unlike the situation in ventricular cells, activation of a chloride conductance in other areas of the heart, even with a physiological Cl\(^-\) gradient, would be expected to have a more profound effect on resting potential. This might then provide an additional mechanism for regulation of pacemaker activity in the heart. Again, this fits well with the early observations of Carmeliet (1961) and Hutter and Noble (1961) suggesting that a Cl\(^-\) current plays a role in diastolic depolarization in Purkinje fibers. However, unlike atrial and pacemaker cells, the resting K+ conductance in Purkinje cells resembles that in ventricular cells (Shah et al., 1987). For this reason, the speculation of a role for a Cl\(^-\) current in pacemaker activity must also take into account ISO-induced changes in other currents, such as the pacemaker current, $I_p$ (DiFrancesco, 1985).

In ventricular cells, $I_C$ does not appear to play a significant role under unstimulated conditions (e.g., in the absence of ISO). This is supported by at least two lines of evidence. First, in the absence of ISO there is very little current elicited by voltage-clamp steps to very depolarized potentials (see Fig. 5). The current that is observed is likely to represent a true leak current. If this current were carried through Cl\(^-\) channel, and these channels were regulated by the second messenger cAMP, then, like other currents regulated by cAMP (e.g., $I_{Ca}$ and $I_h$) one would expect to see some rundown or washout of the current with time. This was not the case. Second, if there were some resting conductance to Cl\(^-\), then in experiments in which $E_{Cl}$ was very positive (e.g., 0 or +15 mV), the resting membrane potential would be expected to be depolarized in the absence of ISO. This also was not the case. Therefore, it appears that the guinea pig ventricular myocyte membrane is only significantly permeable to Cl\(^-\) under conditions where intracellular cAMP levels are elevated. This conclusion has recently been confirmed in similar studies by Bahinski et al. (1989).

**Action Potentials and $I_C$**

The role of a Cl\(^-\) current in repolarization of the cardiac action potential was first suggested by Carmeliet (1961) and Hutter and Noble (1961). However, no macroscopic current or single ion channel could be found to corroborate this hypothesis. Whether or not the current we have identified could be responsible for those previous findings is yet to be determined. One problem with such a conclusion is the apparent necessity for elevated levels of cAMP in our isolated myocytes, whereas the Cl\(^-\)-dependent changes of the action potential in Purkinje fibers were observed under apparently unstimulated conditions. However, existence of endogenous cate-
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Cholamines in vitro multicellular preparations (Vincenzi and West, 1963) could have activated a Cl⁻ current.

The autonomically regulated Cl⁻ current, if found in human cardiac tissue, would undoubtedly be of clinical significance, since the heart is continuously under the influence of adrenergic stimulation. This might then explain some of the antiarhythmic properties of β-adrenergic receptor antagonists, in particular their ability to prolong action potential repolarization (Ijzerman and Soudijn, 1989). Furthermore, it may be that some of the action potential shortening associated with hypoxia and ischemia may be related to an augmentation of I_Ca, since catecholamines are known to be released under these conditions (Videback et al., 1972; Karawatowska-Krynska and Beresewicz, 1983).

Sodium Dependence of I_Ca

There are at least two indirect mechanisms that might be responsible for the apparent Na⁺ sensitivity of I_Ca. When extracellular Na⁺ is removed both the Na/Ca exchanger and the Na/H exchanger will be suppressed. Inhibition of the former would lead to an increase of intracellular Ca²⁺, but it would seem unlikely that this mechanism would be responsible for the attenuation of I_Ca because the intracellular Ca²⁺ concentration in our experiments was maintained at a very low level by blocking the Ca²⁺ current as well as dialyzing the cells with 5 mM EGTA. Furthermore, even if there were changes in intracellular Ca²⁺, this would not be expected to inhibit the chloride conductance because Ca²⁺ has only been shown to activate Cl⁻ currents (Barish, 1983; Gogelein, 1988; Byrne and Large, 1987, 1988; Pacaud et al., 1989).

Removal of extracellular Na⁺ would also be expected to inhibit Na/H exchange, which could lead to an intracellular acidification since these experiments were performed in a bicarbonate-free environment. A similar mechanism has been shown to reduce the conductance of I_K in isolated ventricular myocytes (Harvey and Ten Eick, 1989). However, if this were true, it should be possible to observe a direct pH dependence of I_Ca by exposing cells to external solutions with a reduced pH. When external pH was lowered from 7.4 to 6.5, there was no change in the magnitude of I_Ca (Harvey, R. D., and J. R. Hume, unpublished observation). This suggests that the Na⁺ sensitivity of the Cl⁻ current in guinea pig ventricular myocytes may be a direct effect of Na⁺ ions on the current at some regulatory site on the external surface of the cell membrane.

Comparison with Other Cl⁻ Currents

Although the finding of a macroscopic Cl⁻ current in cardiac muscle is quite new, Cl⁻ currents have been reported in many different tissues including various nerve and epithelial preparations as well as other types of muscle. Skeletal muscle exhibits a large Cl⁻ conductance, and the single channels underlying this conductance have been studied in some detail (Blatz and Magleby, 1984, 1986). Chloride currents have also been observed in some smooth muscle preparations (Byrne and Large, 1987, 1988; Soejima and Kokubun, 1988; Pacaud et al., 1989). While no cAMP dependence of these Cl⁻ currents has been reported so far, they are regulated by intracellular Ca²⁺. While the characteristics of the Cl⁻ currents found in the various
types of muscle seem rather disparate, they are actually quite representative of the different properties of Cl\(-\) conductances found in nonmuscle preparations. Many different Cl\(-\) currents exhibit a dependence on intracellular Ca\(^{2+}\) (Barish, 1983; Mayer, 1985; Gogelein, 1988), and the apparent cAMP dependence of the Cl\(-\) current in cardiac muscle is similar to the Cl\(-\) conductances found in many types of secretory epithelium (Gogelein, 1988).

The regulation of chloride conductances have been most extensively studied in various epithelial preparations. Like the Cl\(-\) current observed in smooth muscle, many, but not all, epithelial Cl\(-\) channels are also activated by intracellular Ca\(^{2+}\). \(I_{cl}\) in heart does not appear to require intracellular Ca\(^{2+}\), because in all of our experiments the cells were dialyzed with 5 mM EGTA and \(I_{ca}\) was blocked. However, this does not rule out the possibility that internal Ca\(^{2+}\) may be involved in regulating \(I_{cl}\) under more physiological conditions. The ISO-induced current in the ventricular myocytes is also like other Cl\(-\) currents in that it could be blocked by the carboxylic acid derivative 9-AC.

The rectifying nature of \(I_{cl}\) observed when external Cl\(-\) was low (<40 mM) is similar to that reported in some epithelial preparations (Gogelein, 1988). It is possible that the rectification of \(I_{cl}\) in cardiac myocytes is due to block of inward current (outward Cl\(-\) movement) by some other ion, a mechanism known to be responsible for rectification of K\(^{+}\) channels (Matsuda et al., 1987; Vandenberg, 1987). However, the fact that the \(I_{cl}\) current-voltage relationship was linear when intra- and extracellular Cl\(-\) concentrations were equal suggests that rectification of the macroscopic current was a consequence of the asymmetrical Cl\(-\) gradient (Goldman, 1943; Hodgkin and Katz, 1949).

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