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A Novel Anionic Inward Rectifier in Native Cardiac Myocytes

Dayue Duan, Lingyu Ye, Fiona Britton, Burton Horowitz, Joseph R. Hume

Abstract—Although the cationic inward rectifiers (Kir and hyperpolarization-activated I_f channels) have been well characterized in cardiac myocytes, the expression and physiological role of anionic inward rectifiers in heart are unknown. In the present study, we report the functional and molecular identification of a novel chloride (Cl^-) inward rectifier (Cl.ir) in mammalian heart. Under conditions in which cationic inward rectifier channels were blocked, membrane hyperpolarization (−40 to −140 mV) activated an inwardly rectifying whole-cell current in mouse atrial and ventricular myocytes. Under isotonic conditions, the current activated slowly with a biexponential time course (time constants averaging 179.7±23.4 [mean±SEM] and 2073.6±287.6 ms at −120 mV). Hypotonic cell swelling accelerated the activation and increased the current amplitude whereas hypertonic cell shrinkage inhibited the current. The inwardly rectifying current was carried by Cl^- ($I_{Cl.ir}$) and had an anion permeability sequence of $Cl^- > I^- \gg$aspartate. $I_{Cl.ir}$ was blocked by 9-anthracene-carboxylic acid and cadmium but not by stilbene disulfonates and tamoxifen. A similar $I_{Cl.ir}$ was also observed in guinea pig cardiac myocytes. The properties of $I_{Cl.ir}$ are consistent with currents generated by expression of ClC-2 Cl^- channels. Reverse transcription polymerase chain reaction and Northern blot analysis confirmed transcriptional expression of ClC-2 in both atrial and ventricular tissues and isolated myocytes of mouse and guinea pig hearts. These results indicate that a novel $I_{Cl.ir}$ is present in mammalian heart and support a potentially important role of ClC-2 channels in the regulation of cardiac electrical activity and cell volume under physiological and pathological conditions. The full text of this article is available at http://www.circresaha.org. **(*Circ Res*. 2000;86:e63-e71.)**

Key Words: channel, Cl^- ■ action potential ■ cell volume

At least six distinct types of sarcolemmal Cl^- currents have been functionally identified in cardiac myocytes.[1–5] These include Cl^- currents activated by intracellular cAMP-PKA ($I_{Cl.PKA}$), PKC ($I_{Cl.PKC}$), intracellular Ca^{2+} ($I_{Cl.Ca}$ or I_{to2}), extracellular ATP ($I_{Cl.ATP}$), cell volume ($I_{Cl.vol}$), and a basally active Cl^- current ($I_{Cl.b}$). It has been well established that $I_{Cl.PKA}$ is mediated by cardiac expression of an isoform of the epithelial cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel,[6–8] and recent data suggest that $I_{Cl.vol}$ and $I_{Cl.b}$ may be attributed to cardiac expression of a member of the ClC Cl^- channel superfamily,[9] ClC-3.[10,11] Functional data suggest that $I_{Cl.PKC}$ and $I_{Cl.ATP}$ may also be attributed to activation of CFTR Cl^- channels.[12–16] Although the molecular entity responsible for $I_{Cl.Ca}$ remains to be determined, it may be encoded by a member of the new CLCA gene family.[17] With physiological Cl^- gradients, all of these Cl^- currents activate predominantly at depolarized voltages, exhibit outward rectification, and thus contribute significantly to shortening of action potential duration; although activation of $I_{Cl.Ca}$ under conditions of intracellular Ca^{2+} overload may, in addition, contribute to the development of oscillatory delayed afterdepolarizations.[3,5]

Recently, another member of the ClC Cl^- channel family, ClC-2, has been cloned originally from rat heart and brain[18] and then from rabbit heart.[19] Although it has been shown that functional expression of rat ClC-2 (rClC-2) and rabbit cardiac ClC-2 (ClC-2α) mRNA in *Xenopus* oocytes gives rise to an inwardly rectifying, hyperpolarization-activated Cl^- conductance that is modulated by changes in cell volume and extracellular pH,[18–20] inwardly rectifying hyperpolarization-activated Cl^- currents with properties resembling ClC-2 have yet to be identified in native cardiac myocytes. However, hyperpolarization-activated ClC-2 channels expressed in heterologous expression systems bear a striking resemblance to the well-characterized hyperpolarization-activated cationic pacemaker current, I_f (or I_h), found in many mammalian cardiac cell types[21,22] and are now known to belong to the HCN gene family.[23–25] Because of similarities in inward rectification and relatively slow activation during membrane hyperpolarization, it is possible that coexpression of ClC-2 Cl^- channels in some native cardiac myocytes, which also express I_f (HCN), may in part explain earlier observations that suggested some anion sensitivity of the hyperpolarization-activated I_f.[26–28]

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In the present study, using both electrophysiological and molecular biological techniques, we tested the hypothesis that endogenous expression of ClC-2 may be responsible for a novel, inwardly rectifying hyperpolarization-activated anion conductance in native atrial and ventricular myocytes isolated from mouse and guinea pig hearts. The data demonstrate that, under conditions during which cationic inwardly rectifying channels are blocked or eliminated, inwardly rectifying currents with an anion permeability of $Cl^- > I^- \gg$ aspartate (Asp^-) can be detected in a percentage of mouse and guinea pig atrial and ventricular myocytes. The biophysical and pharmacological properties of this inwardly rectifying Cl^- current ($I_{Cl.ir}$) are nearly identical to the known properties of cloned ClC-2 Cl^- channels expressed in heterologous expression systems. Finally, we present evidence using the reverse transcription–polymerase chain reaction (RT-PCR) and Northern blot analysis that confirms transcriptional expression of a ClC-2 homologue in both atrial and ventricular tissue and cells from mouse and guinea pig heart. These results provide the first evidence that a novel $I_{Cl.ir}$, which may be encoded by ClC-2, is functionally expressed in mammalian heart. $I_{Cl.ir}$, like cationic inward rectifiers, may play an important role in the regulation of action potential duration, resting membrane potential, and pacemaker activity under both physiological and pathophysiological conditions. A preliminary report describing these results has been published.[29]

Materials and Methods

Electrophysiological Measurements

Whole-cell currents were measured from single atrial and ventricular myocytes enzymatically isolated from mouse and guinea pig hearts using the tight-seal, whole-cell, voltage-clamp technique,[30] as previously described.[16,31] To prevent contamination from Ca^{2+} and K^+ currents, and the cationic inward rectifiers I_{K1} and I_f, nisoldipine (1 μmol/L), 4-aminopyridine (4-AP, 2 mmol/L), Ba^{2+} (2 mmol/L), and Cs^+ (10 mmol/L) were present continuously in the extracellular bath solutions and cations in the intracellular pipette solution and, in some experiments, in the bath solution were replaced by the large impermeant cation, *N*-methyl-D-glucamine (NMDG). Hypotonic (230 mOsm/kg H_2O, measured by freezing-point depression) bath solutions contained (mmol/L) NaCl 100, $MgCl_2$ 1, $CaCl_2$ 1, $BaCl_2$ 2, NaH_2PO_4 0.33, CsCl 10, HEPES 10, glucose 5.5, $[Cl^-]_o$ 118; pH 7.4. In some experiments, NaCl was replaced with equimolar concentrations (100 mmol/L) of NaI or Na-aspartate. The isotonic and hypertonic bath solutions were the same as the hypotonic solution except the osmolarity was adjusted to 290 mOsm/kg H_2O and 360 mOsm/kg H_2O by adding mannitol. The pipette solution contained (in mmol/L) NMDG-Cl 118, MgATP 5, NaGTP 0.1, ethylene-glycobis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 5, HEPES 5, $[Cl^-]_i$ 118; pH 7.4, 290 mOsm/kg H_2O using mannitol. In low $[Cl^-]_i$ (20 mmol/L) pipette solution, 98 mmol/L NMDG-Cl was replaced by NMDG-aspartate. A bridge (3 mol/L KCl in agar salt) between the bath and a Ag/AgCl reference electrode immersed in pipette solution was used to minimize changes in liquid junction potential, and junction potentials were zeroed before formation of the membrane-pipette seal. Cell dimensions (length and width) were roughly estimated with a calibrated graticule in the microscope eyepiece. Experiments were conducted at room temperature (22°C to 24°C).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RT-PCR of total RNA prepared from cardiac tissues using the Trizol reagent (Life Technologies) or from enzymatically dispersed individual cardiac myocytes using SNAP total RNA isolation kit (Invitrogen) was performed as previously described.[10] The primer region (forward: 5′-TGGGAGGAGCAGCAGCTGAA-3′, reverse: 5′-CAGAGTGCATGCACCTCT-GTGGT-3′) is specific for rClC-2 (GenBank accession No. X64139)[20] and corresponds to nucleotides 2515 to 2822, generating a 307-nucleotide amplification product. Automatic nucleotide sequencing was performed on both strands using the dideoxy nucleotide chain termination method (Genetic Analyzer, Model 310, Perkin Elmer).

Northern Blot Analysis

To confirm the expression of ClC-2 in cardiac tissues, Northern blot analysis was performed as described previously.[10,16] A 420-bp rClC-2 cDNA probe was radiolabeled with ^{32}P by random priming.[32] Hybridization was performed under the same conditions overnight. The filters were washed at high stringency (3 times in 2× SSC at room temperature for 5 minutes then twice in 0.2× SSC/0.1% SDS at 65°C for 30 minutes) to ensure specificity of labeling. Filters were exposed to film, and autoradiography was performed using a BioRad phosphoimager (Hercules).

Data Analysis

Data are presented as mean±SEM. Student's *t* test or ANOVA with Scheffé contrasts was used to determine statistical significance. A two-tailed probability (*P*) of ≤5% was considered significant.

Results

Novel Volume-Regulated Inward Rectifier Cl^- Current ($I_{Cl.ir}$) in Mouse Heart

With the cationic inward rectifier blockers (Cs^+ 10 mmol/L and Ba^{2+} 2 mmol/L) in the extracellular bath solution and a large impermeable cation NMDG in the intracellular pipette solution to replace all permeable cations, hyperpolarization of the cell membrane activated a time-dependent inward current in both atrial and ventricular myocytes isolated from mouse heart. Figure 1A shows an example of the currents recorded from ventricular myocytes under isotonic, hypotonic, and hypertonic conditions. The current activated on hyperpolarization from −40 to −140 mV under isotonic (290 mOsm/kg H_2O) conditions. Subsequent exposure of the same cells to hypotonic (230 mOsm/kg H_2O, 21% hypotonic) bath solutions caused significant cell swelling and caused a further increase in current amplitude (Figure 1). Overall, cell areas during hypotonic cell swelling were estimated to increase from 1633±165 to 2234±176 μm^2 (n=5, $P<0.05$). Hypotonic cell swelling not only increased current amplitudes but also accelerated the kinetics of current activation. As shown in Figure 1B, the time-dependent activation of the current at hyperpolarized potentials was relatively slow in onset and could be best fit by a biexponential function. Under isotonic conditions (left panel of Figure 1B), the mean activation time constants at −120 mV were τ_1=179.7±23.4 ms and τ_2=2073.6±287.6 ms (n=5). Under hypotonic conditions (right panel of Figure 1B), both the fast activation time constant (τ_1=97.5±8.5 ms at −120 mV, n=5, P=0.011 versus isotonic condition) and the slow activation time constant (τ_2=656.4±113.6 ms at −120 mV, P=0.002 versus isotonic condition) were significantly reduced. Hypertonic cell shrinkage caused inhibition of the current (Figures 1A and 1C). Under isotonic conditions, we observed similar hyperpolarization-activated currents in 5 of 52 (9.6%) ventricular myocytes, which was further regulated by hypotonic

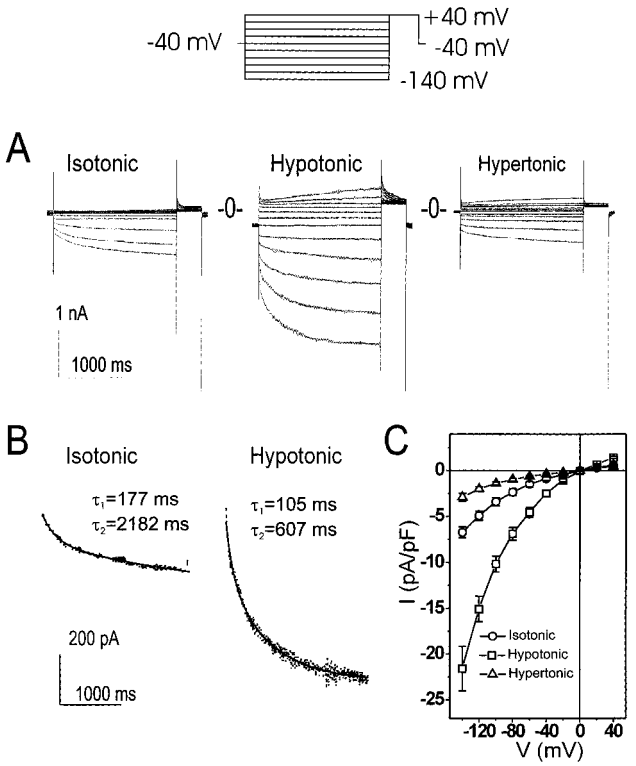

Figure 1. Hyperpolarization-activated anion current and its sensitivity to cell volume in mouse ventricular myocytes. Currents were recorded using voltage-clamp protocols shown on top. Cells were held at −40 mV, and test potentials were applied from −140 to +40 mV in +20-mV increments for 2 seconds and then to +40 mV for 400 ms before return to the holding potential. The test potentials were applied at an interval of 10 seconds (insert on the top). A, Hyperpolarization voltage pulses activated inward currents under isotonic conditions (left panel). Subsequent exposure of the same cell to hypotonic solution (0.79T) caused a further increase in current amplitudes (middle panel). Further exposure of the cell to hypertonic solution caused a decrease in current amplitudes (right panel). B, Effects of hypotonic cell swelling on the time course of activation of the inward rectifying current. Representative current recordings from one cell after hyperpolarization to −120 mV under isotonic (left panel) and hypotonic (right panel) conditions, respectively, are shown. The points represent current activation data, and the solid lines are least-square curve fits obtained with the curve-fitting program Clampfit (Axon Instruments). The activation process was best fit to a biexponential function with a fast time constant (τ_1) of 177 ms and a slow time constant (τ_2) of 2182 ms under isotonic conditions. Hypotonic cell swelling increased the amplitude of the current and also accelerated the activation kinetics (τ_1=105 ms, τ_2=607 ms). C, Mean *I*-V relationship from 5 different cells under isotonic (○), hypotonic (□), and hypertonic (△) conditions. Currents were measured at the end of each 2-second test pulse. Mean reversal potentials (E_{rev}) of the currents under isotonic, hypotonic, and hypertonic conditions were 2.0±3.8 mV, 3.3±3.4 mV, and −1.7±3.3 mV (n=5, *P*=NS), respectively, which were very close to the predicted equilibrium potential of Cl^- (E_{Cl}=0 mV) with a symmetrical Cl^- gradient ($[Cl^-]_o/[Cl^-]_i$=118/118 mmol/L).

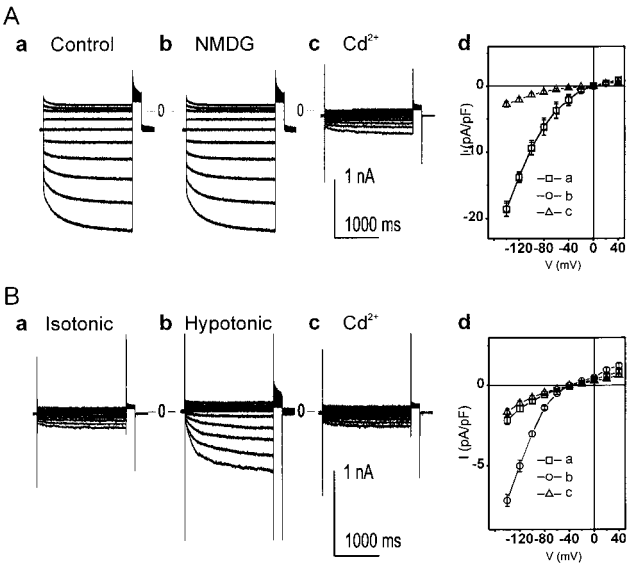

Figure 2. Ion selectivity of hyperpolarization-activated currents in mouse ventricular myocytes. Currents were recorded using voltage-clamp protocols shown on top of Figure 1. A-a, Current tracings recorded from a mouse ventricular myocyte in standard hypotonic bath solution (Control), which contained Na^+, Ba^{2+}, Ca^{2+}, and Cs^+ (see Materials and Methods). A-b, Current tracings recorded from the same cell after exposure to hypotonic solution in which all cations were replaced with an impermeable large cation NMDG. A-c, Further exposure of the cell to hypotonic NMDG solution that contained 0.3 mmol/L cadmium (Cd^{2+}) caused a decrease in current amplitudes. A-d, Mean *I*-V relationship from 3 different cells under hypotonic control (□), hypotonic NMDG (○), and hypotonic NMDG+Cd^{2+} (△) conditions. Substitution of cations in extracellular bath solution failed to alter the *I*-V relationship and reversal potential (−1.2±3.4 mV vs 0.4±2.4 mV, n=3, *P*=NS) of the inwardly rectifying currents. B-a, Current tracings recorded from a mouse ventricular myocyte in standard isotonic bath solution (Isotonic) with Cl^- in intracellular pipette solution partially replaced by Asp^- (total $[Cl^-]_i$=20 mmol/L). B-b, Current tracings recorded from the same cell after exposure to standard hypotonic solution (Hypotonic). B-c, Further exposure of the cell to hypotonic solution that contained 0.3 mmol/L (Cd^{2+}) caused a decrease in current amplitudes. B-d, Mean *I*-V relationship from 4 different cells under isotonic (□), hypotonic (○), and hypotonic Cd^{2+} (△) conditions. Compared with the currents recorded with high (118 mmol/L) $[Cl^-]_i$ (see panel A and Figure 1), reduction in $[Cl^-]_i$ reduced the current density of the hypotonic cell swelling–activated inward current and shifted the reversal potential of the current to a more negative potential (−43.8±1.6 mV, n=4), which is very close to the predicted E_{Cl} (−45.6 mV).

cell swelling and hypertonic cell shrinkage. Figure 1C shows the mean current-voltage (*I*-V) relationship of the hyperpolarization-activated inward currents under isotonic (○), hypotonic (□), and hypertonic (△) conditions. These currents had a strong inwardly rectifying *I*-V relationship with mean reversal potentials (E_{rev}) of 2.0±3.8 mV, 3.3±3.4 mV, and −1.7±3.3 mV (n=5, *P*=NS), under isotonic, hypotonic, and hypertonic conditions, respectively, which were very close to the predicted equilibrium potential of Cl^- (E_{Cl}=0 mV) with a symmetrical Cl^- gradient ($[Cl^-]_o/[Cl^-]_i$=118/118 mmol/L).

As shown in Figure 2A, the time-dependent inwardly rectifying currents in mouse ventricular myocytes remained unchanged when all cations in the extracellular solution were also replaced by NMDG. These currents, however, were significantly reduced by extracellular cadmium (0.3 mmol/L), which has been shown to block inwardly rectifying Cl^- current in noncardiac cells.[33,34] Furthermore, as shown in Figure 2B, when $[Cl^-]_i$ was partially substituted with equimolar Asp^- (98 mmol/L; $[Cl^-]_i$ 20 mmol/L), the hypotonic cell swelling–activated inward current was significantly smaller than the current recorded with high $[Cl^-]_i$ (see Figures 1 and 2A), and the reversal potential of the current shifted to more

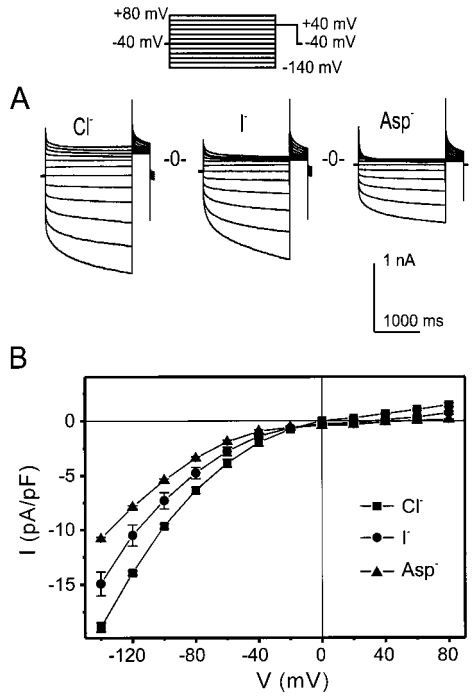

Figure 3. Anion selectivity of hypotonic cell swelling–activated $I_{Cl.ir}$ in mouse atrial myocytes. A, Representative whole-cell currents recorded in high extracellular Cl^- ($[Cl^-]_o$=118 mmol/L) bath solution (left), $[Cl^-]_o$ were reduced to 18 mmol/L (E_{Cl}48.3 mV) by equimolar (100 mmol/L) replacement of Cl^- with I^- (middle) or Asp^- (right). Currents were recorded using voltage-clamp protocols shown on top. Cells were held at −40 mV, and test potentials were applied from −140 to +80 mV in +20-mV increments for 2 seconds and then to +40 mV for 400 ms before return to the holding potential. The test potentials were applied at an interval of 10 seconds. B, Mean *I*-V relationships of swelling-activated $I_{Cl.ir}$ before (■) and after $[Cl^-]_o$ was substituted by I^- (●) or Asp^- (▲). I^- and Asp^- substitution of Cl^- shifted the reversal potential of $I_{Cl.ir}$ from +3.5±0.7 mV (Cl^-) to +33.4±4.6 mV (I^-) and +47.2±1.2 mV (Asp^-) (n=4), respectively.

negative potential with a mean value of −43.8±1.6 mV (n=4), which is very close to the predicted E_{Cl} (−45.5 mV). Again, these currents were significantly blocked by extracellular cadmium. These results strongly indicate that the current in mouse ventricular myocytes is neither I_f nor a swelling-induced nonspecific cationic inward rectifier current.[35,36] It may represent a novel volume-regulated Cl^- inward rectifier current, $I_{Cl.ir}$.

Similar $I_{Cl.ir}$ was also observed in mouse atrial myocytes (16 of 113, 14.2%). The relative anion selectivity of $I_{Cl.ir}$ was further examined by replacing $[Cl^-]_o$ with an equimolar concentration (100 mmol/L) of iodide (I^-) or aspartate (Asp^-) (E_{Cl}48.3 mV) under hypotonic conditions. Cells were held at −40 mV, and test potentials were applied at an interval of 10 seconds from −140 mV to +80 mV for 2 seconds in +20-mV increments and then to +40 mV for 400 ms before return to the holding potential (see Figure 3 and inset). In four different mouse atrial cells, reduction of $[Cl^-]_o$ caused a shift of E_{rev} of the current from +3.5±0.7 mV (Cl^-) to +33.4±4.6 mV (I^-) and +47.2±1.2 mV (Asp^-), respectively. The permeability ratios (permeability ratio of anion X with respect to Cl^-, P_x/P_{Cl}) were then calculated from the shifts of E_{rev} using the modified Goldman-Hodgkin-Katz equation.[37]

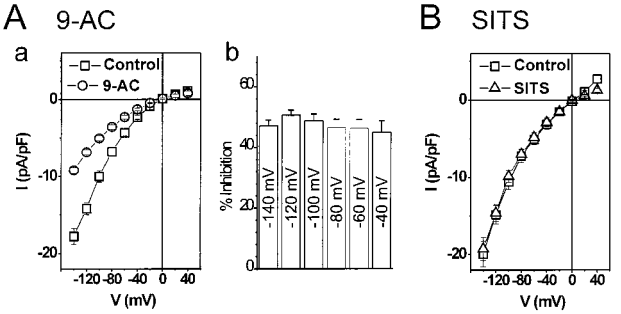

Figure 4. Effects of Cl^- channel blockers on $I_{Cl.ir}$. Currents were recorded using the same protocol as shown in Figure 1. A, Carboxylic acid derivative 9-AC blocked $I_{Cl.ir}$ in mouse atrial myocytes. A-a, Mean *I*-V curves of $I_{Cl.ir}$ obtained from 4 different mouse atrial cells under hypotonic control condition (□) and after 1 mmol/L 9-AC (○). A-b, Percentage of current inhibition by 9-AC at various test potentials. The inhibition of the current by 9-AC showed no voltage dependence. B, Mean *I*-V curves of $I_{Cl.ir}$ obtained from 5 different mouse atrial cells under hypotonic control condition (□) and after exposed to 1 mmol/L SITS (△). The disulfonic stilbene derivative SITS failed to affect $I_{Cl.ir}$ in mouse atrial myocytes.

The cell swelling–activated inward rectifier channel had an estimated P_I/P_{Cl} of 0.22±0.09 (n=4) and P_{Asp}/P_{Cl} of 0.04±0.01 (n=4), suggesting that the current is conducted through an anion selective channel with a relative anion permeability of $Cl^- > I^- \gg Asp^-$.

Effects of Cl^- Channel Blockers on $I_{Cl.ir}$

Several compounds including arylaminoalkyl benzoates derivatives such as 9-anthracene-carboxylic acid (9-AC) and disulfonic stilbene derivatives such as 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) have been identified as effective blockers of a variety of cardiac[5,38,39] and noncardiac Cl^- channels.[40] Therefore, we assessed the effects of 9-AC and SITS on the swelling-induced $I_{Cl.ir}$ in mouse atrial myocytes. As shown in Figure 4A, 1 mmol/L of 9-AC significantly ($P<0.001$ versus control at all test potentials) blocked $I_{Cl.ir}$ (Figure 4A-a) in a voltage-independent manner (Figure 4A-b). The inhibition of the current amplitude at test potentials of −140, −120, −100, −80, −60, and −40 mV was 47.1±1.9%, 50.6±1.7%, 48.8±2.2%, 46.6±2.7%, 46.3±2.9%, and 45.0±3.8%, respectively (n=4, P=NS for voltage dependence). In contrast, $I_{Cl.ir}$ was not sensitive to disulfonic stilbene derivatives. As shown in Figure 4B, 1 mmol/L SITS failed to affect the current in mouse atrial cells. The changes in current densities after SITS were −3.0±1.7%, −1.3±2.6%, −6.2±4.4%, −2.3±3.5%, −4.2±1.6%, and −5.4±4.0% at test potentials of −140, −120, −100, −80, −60, and −40 mV, respectively (n=5, P=NS versus control at all test potentials).

$I_{Cl.ir}$ in Guinea Pig Cardiac Myocytes

Hyperpolarization-activated inwardly rectifying, volume-sensitive, stilbene-insensitive currents with properties similar to mouse cardiac $I_{Cl.ir}$ were also observed in some guinea pig atrial (6 of 56, 10.7%) and ventricular (3 of 32, 9.4%) myocytes. Under hypotonic conditions, it has been reported that an outwardly rectifying volume-regulated Cl^- current ($I_{Cl.vol}$) is present in ≈90% of atrial myocytes and ≈30% of

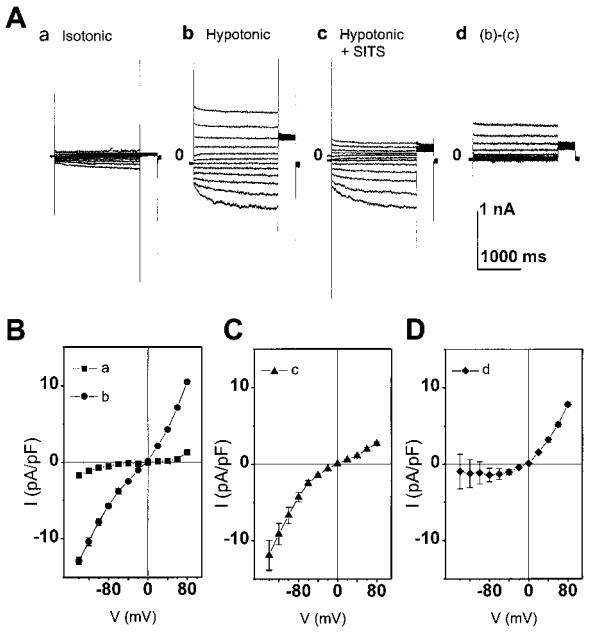

Figure 5. Volume-regulated whole-cell anion currents in guinea pig ventricular myocytes. Currents were recorded using the same protocols as shown in Figure 3. A, Representative whole-cell currents recorded under isotonic (A-a), hypotonic (A-b), and hypotonic 1 mmol/L SITS (A-c) conditions. Panel A-d shows the SITS-sensitive difference current obtained by subtracting the current in panel A-c from the current in panel A-b. SITS inhibited mainly the volume-regulated, outwardly rectifying Cl^- current ($I_{Cl.vol}$; see text). B, Mean *I*-V curves of whole-cell currents obtained from 3 different guinea pig ventricular myocytes under isotonic (■) and hypotonic (●) conditions. C, Mean *I*-V curve of SITS-insensitive whole-cell currents, which exhibited strong inward rectification. D, Mean *I*-V curve of SITS-sensitive currents, which exhibited strong outward rectification.

ventricular myocytes of guinea pig heart.[31,41–44] Different from $I_{Cl.ir}$, however, $I_{Cl.vol}$ is outwardly rectifying, deactivates at positive potentials, has an anion permeability sequence of $I^- > Cl^-$, and is sensitive to disulfonic stilbene (such as SITS) Cl^- channel blockers.[31,41,42,44] Figure 5A shows an example of whole-cell currents recorded from a guinea pig ventricular myocyte, in which both $I_{Cl.vol}$ and $I_{Cl.ir}$ were activated under hypotonic conditions. In these experiments, currents were recorded using the same bath and pipette solutions as in Figure 1 and the same voltage-clamp protocol as in Figure 3. As shown in Figure 5A-a, small hyperpolarization-activated time-dependent inward currents could be detected in this cell even under isotonic conditions. Subsequent hypotonic cell swelling not only increased the inward currents but also activated large outward currents (Figure 5A-b). Although the outward currents showed time-dependent deactivation at positive depolarizing potentials, the inward currents showed time-dependent activation at negative membrane potentials. Subsequent exposure of the same cell to 1 mmol/L of SITS, an effective blocker of cardiac $I_{Cl.vol}$, caused an inhibition of mainly the outward current (Figure 5A-d), leaving the time-dependent inward current largely unaffected (Figure 5A-c). Similar results were observed in three guinea pig ventricular myocytes, and the mean *I*-V curves under isotonic (■), hypotonic(●), and hypotonic 1 mmol/L SITS (▲) conditions are shown in Figures 5B and 5C, respectively. The SITS-sensitive current showed outward rectification and deactivation at more positive potentials (Figures 5A-d and 5D), which are typical features of $I_{Cl.vol}$.[10,31,42] The SITS-insensitive inwardly rectifying current (Figures 5A-c and 5C), however, had properties very similar to those of $I_{Cl.ir}$ observed in mouse cardiac myocytes described earlier in Figure 1. The estimated current densities for guinea pig $I_{Cl.ir}$ in ventricular myocytes (-10.4 ± 0.5 pA/pF, at -120 mV, n=3) and atrial myocytes (-12.8 ± 0.7 pA/pF, at -120 mV, n=3) revealed no significant differences (P=NS).

We also examined the Cl^- dependence of the current in guinea pig atrial myocytes using different $[Cl^-]_i$. As shown in Figure 6A-a, when $[Cl^-]_i$ was reduced to 20 mmol/L by replacing NMDG-Cl with equimolar amount (98 mmol/L) of NMDG-aspartate, hypotonic cell swelling induced an activation of both small slowly activating inward currents and large outward currents (Figure 6A-a). Subsequent exposure of the same cell to the $I_{Cl.ir}$ blocker Cd^{2+} (0.3 mmol/L) caused an inhibition of mainly the inward currents (Figures 6A-b and 6A-d). Further exposure of the cell to 10 μmol/L of tamoxifen (TMX), which blocks $I_{Cl.vol}$ in many cardiac and noncardiac cells[10,11,33,41] but has no effect on $I_{Cl.ir}$ in noncardiac cells,[33] caused a further inhibition of the currents (Figures 6A-c). Similar results were observed in three guinea pig atrial myocytes, and the mean *I*-V curves under hypotonic control (■), hypotonic+0.3 mmol/L Cd^{2+}(●), and hypotonic+0.3 mmol/L Cd^{2+}+10 μmol/L TMX (▲) conditions, respectively, are shown in Figure 6B. The Cd^{2+}-sensitive currents showed time-dependent activation at hyperpolarization potentials (Figure 6A-d) and had an inwardly rectifying *I*-V relationship with a mean reversal potential of -42.6 ± 4.4 mV (n=3), which is very close to the estimated E_{Cl} (-45.6 mV). These properties are very similar to those of $I_{Cl.ir}$ observed in mouse cardiac myocytes under the same conditions (see Figure 2B). The TMX-sensitive currents (Figures 6A-e and 6D) were outwardly rectifying and had typical properties of $I_{Cl.vol}$ in the same tissue described previously.[10,31,42] These results suggest that, in addition to the outwardly rectifying $I_{Cl.vol}$, hypotonic cell swelling also activates an inwardly rectifying $I_{Cl.ir}$ in these guinea pig cardiac myocytes.

Molecular Expression of ClC-2 in Mouse and Guinea Pig Heart

The biophysical and pharmacological properties of $I_{Cl.ir}$ in mouse and guinea pig heart described above, including the anion selectivity, inward rectification, regulation by cell volume, sensitivity to 9-AC and Cd^{2+}, and insensitivity to SITS and TMX, are nearly identical to those known properties of currents generated by ClC-2 channels expressed in *Xenopus* oocytes[19,20,45] and mammalian HEK 293 cells,[46] suggesting that ClC-2 is a strong molecular candidate that may be responsible for $I_{Cl.ir}$. Therefore, we tested for molecular expression of ClC-2 in mouse and guinea pig heart.

Figure 7A shows an agarose gel depicting a ClC-2–specific RT-PCR product generated from RNA derived from mouse atrial and ventricular tissues. The RT-PCR reaction of total RNA prepared from both atrial and ventricular tissue with specific primers designed to amplify a 307-nucleotide of

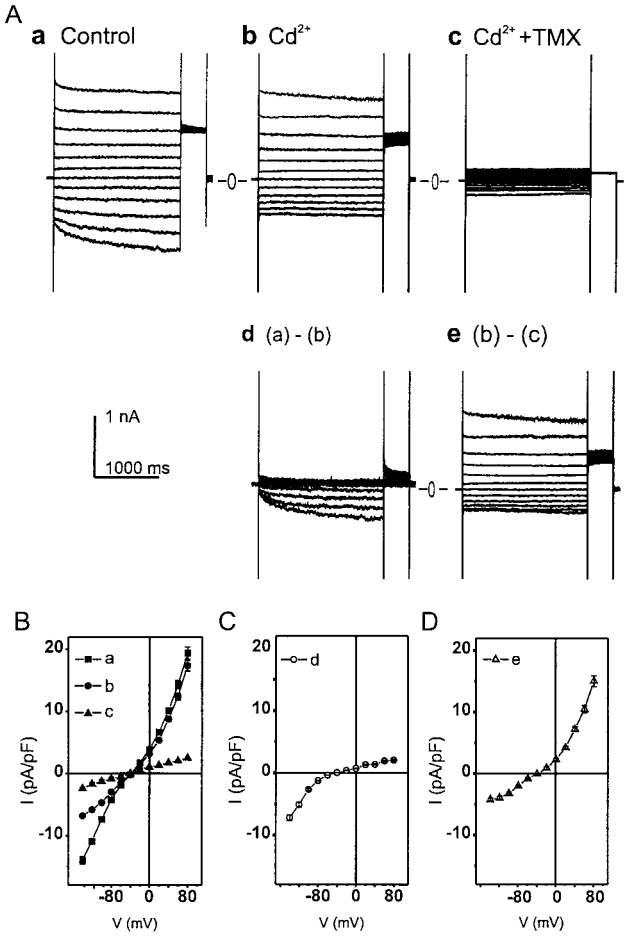

Figure 6. Volume-regulated whole-cell anion currents in guinea pig atrial myocytes. Currents were recorded using the same low Cl^- (20 mmol/L) pipette as shown in Figure 2 and the same protocols as shown in Figure 3. A, Representative whole-cell currents recorded under hypotonic control (A-a), hypotonic 0.3 mmol/L Cd^{2+} (A-b), and hypotonic Cd^{2+} and 10 μmol/L TMX (A-c) conditions. Panel A-d shows the Cd^{2+}-sensitive difference current obtained by subtracting the current in panel A-b from the current in panel A-a. Cd^{2+} inhibited a time-dependent inwardly rectifying current. Panel A-e shows the TMX-sensitive difference current obtained by subtracting the current in panel A-c from the current in panel A-b. TMX inhibited mainly the volume-regulated, outwardly rectifying Cl^- current ($I_{Cl,vol}$; see text). B, Mean *I*-V curves of whole-cell currents obtained from 3 different guinea pig ventricular myocytes under hypotonic (■), hypotonic Cd^{2+} (●), and hypotonic Cd^{2+}+TMX (▲) conditions. C, Mean *I*-V curve of Cd^{2+}-sensitive whole-cell currents shows strong inward rectification and reversed at -42.6 ± 4.4 mV (n=3), which is very close to the estimated E_{Cl} (-45.6 mV). D, Mean *I*-V curve of TMX-sensitive currents exhibits strong outward rectification.

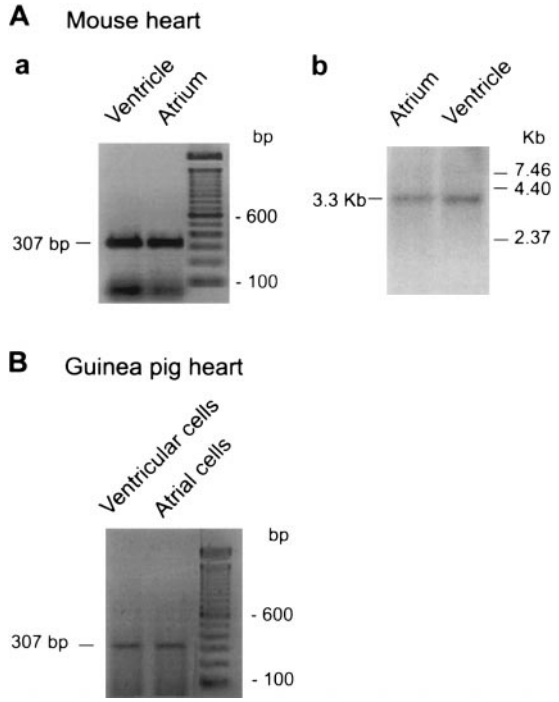

Figure 7. Molecular expression of ClC-2 in mouse heart. A, Agarose gel depicting ClC-2–specific RT-PCR product from mouse atrial and ventricular tissue. The ClC-2–specific primers amplified a 307-nucleotide product of mouse ClC-2 (nucleotide positions 2515 to 2822), which confirmed transcriptional expression of ClC-2 in both atrial and ventricular cells. B, Northern analysis of ClC-2 expression in mouse cardiac tissues. Total RNA from atrial (20 μg) and ventricular (20 μg) tissue was hybridized with a ^{32}P-labeled mouse ClC-2 probe (420 bp) as described in Materials and Methods. The mouse ClC-2 transcript was detected at ≈3.3 kb. C, Agarose gel depicting ClC-2–specific RT-PCR products generated from RNA derived from isolated ventricular and atrial myocytes enzymatically dispersed from guinea pig heart. Only rod-shaped myocytes with clear cross striations and no visible blebs on their surface were used for total RNA isolation. Sequence analysis of the products from RT-PCR of RNA isolated from these myocytes verified that transcripts identical to rClC-2 were present in guinea pig atrial and ventricular myocytes.

rClC-2 (nucleotide positions 2515 to 2822, see Materials and Methods) confirmed the transcriptional expression of ClC-2 in both atrium and ventricle. The RT-PCR product was sequenced and determined to be identical to the previously cloned rClC-2.[20] Northern blot analysis also indicated that ClC-2 mRNA is expressed in both atrial and ventricular tissue from mouse heart. Figure 7B shows hybridization to a transcript of ≈3.3 kb, which is similar in size to ClC-2 mRNA expressed in rat heart.[20] We also amplified a ClC-2–specific RT-PCR product generated from RNA derived from isolated single atrial and ventricular myocytes enzymatically dispersed from guinea pig heart (Figure 7C). This RT-PCR reaction used the same specific primers and amplified a 307-nucleotide product of gpClC-2, confirming transcriptional expression of ClC-2 in single atrial and ventricular myocytes.

Discussion

In the present study, we first characterized the biophysical and pharmacological properties of a volume-regulated inwardly rectifying Cl^- current, $I_{Cl,ir}$, in mammalian heart. In both atrial and ventricular myocytes of mouse and guinea pig heart, $I_{Cl,ir}$ activated slowly on hyperpolarization with a biexponential time course. The current was very sensitive to changes in cell volume. Whereas hypertonic cell shrinkage diminished the current, hypotonic cell swelling increased the current and accelerated the activation kinetics. $I_{Cl,ir}$ exhibited strong inward rectification with a reversal potential close to the estimated E_{Cl} in symmetrical Cl^- and physiological

asymmetrical Cl^- conditions. The anion selectivity sequence of $I_{Cl.ir}$ was $Cl^- > I^- \gg Asp^-$. Although $I_{Cl.ir}$ was strongly blocked by Cd^{2+} and the carboxylic acid derivative, 9-AC, it was not sensitive to inhibition by effective blockers of $I_{Cl.vol}$ such as TMX and the disulfonic stilbene derivative, SITS. All these properties clearly separate $I_{Cl.ir}$ from other types of previously described Cl^- currents in heart, suggesting that $I_{Cl.ir}$ may be mediated by a novel type of Cl^- channel.[5]

ClC-2 belongs to a large ClC gene family of voltage-gated Cl^- channels and is ubiquitously expressed in many tissues.[9,20] Initially, ClC-2 was cloned from rat heart and brain, but only the brain form was subsequently sequenced.[20] It has 907 amino acids, a molecular mass of ≈99 kDa, and shares ≈50% homology with ClC-0 and ClC-1. When transiently expressed in *Xenopus* oocytes, ClC-2 channels activate during hyperpolarization (−90 to −180 mV) and are further stimulated by cell swelling. ClC-2 channels exhibit a strong inwardly rectifying instantaneous *I*-V relationship and an anion permeability of $Cl^- \geq Br^- > I^-$. The channel is blocked by carboxylic acid derivatives and Cd^{2+} but is largely unaffected by disulfonic stilbene derivatives and TMX.[18–20,47] When expressed in mammalian (HEK 293) cells, ClC-2 channels are active under isotonic conditions but exhibit faster activation kinetics than the channel expressed in *Xenopus* oocytes.[46] A rabbit homologue of ClC-2 (ClC-2G) was isolated from a rabbit gastric cDNA library.[45] ClC-2G has also been shown to be ubiquitously expressed in rabbit[19,45] and human[48] tissues. Recently, Furukawa et al[49] isolated a ClC-2α clone from a rabbit heart cDNA library that is identical to ClC-2G. They also proposed that an alternatively spliced, truncated form of ClC-2, ClC-2β, may be specifically expressed in heart. However, in a subsequent study, they found this truncated ClC-2β clone is likely to be an artifact of library construction and not a product of alternative splicing.[19] It has been shown that, when expressed in *Xenopus* oocytes, ClC-2G (ClC-2α) channels have similar biophysical and pharmacological characteristics as rClC-2 channels.[19,45]

Cl^- currents with properties similar to those of heterologously expressed ClC-2 channels have not been specifically identified in native cardiac myocytes before, despite early reports of a hyperpolarization-activated Cl^- current in some multicellular cardiac preparations.[26,28,50] These results have generally been attributed to anion sensitivity of the hyperpolarization-activated I_f due to screening of positive charges near the external pore of the I_f channel[51] However, the hyperpolarization-activated inwardly rectifying currents observed in our experiments are unlikely due to I_f or other cationic inward rectifiers because (1) 10 mmol/L Cs^+ and 2 mmol/L Ba^{2+} were included in the external bath solutions and the nonpermeable large cation NMDG was the only major cation in the internal pipette solutions, effectively precluding possible contamination from I_f and other cationic inward rectifiers, (2) the *I*-V relationship was not altered by replacement of cations with NMDG and shifted as expected for a Cl^- selective channel when intracellular aspartate was substituted for Cl^-, and the measured reversal potentials of $I_{Cl.ir}$ closely corresponded to the predicted value of E_{Cl}, (3) substitution of $[Cl^-]_o$ by small anions such as I^- or Asp^- shifted the reversal potential of $I_{Cl.ir}$ to positive potentials, consistent with a channel with a relative anion permeability of $Cl^- > I^- \gg Asp^-$, and (4) $I_{Cl.ir}$ in cardiac myocytes was blocked by extracellular Cd^{2+}, an effective blocker of $I_{Cl.ir}$ in many noncardiac cells. Recently, Clemo et al[35] have reported a nonspecific cationic inward rectifier current ($I_{Cir.swell}$) that is also regulated by cell volume in rabbit ventricular myocytes and ventricular myocytes isolated from dog with tachycardia-induced congestive heart failure.[36] However, $I_{Cir.swell}$ differs from $I_{Cl.ir}$ in mouse and ventricular myocytes in that $I_{Cir.swell}$ (1) does not show time-dependent activation at hyperpolarization potentials (see Figure 1 in Reference 35), (2) is not sensitive to changes in bath Cl^- concentrations, (3) is not sensitive to 9-AC, and (4) is dependent on extracellular cations and can be abolished by NMDG replacement for extracellular cations. Our results, therefore, clearly exclude the possibility that $I_{Cl.ir}$ is the same as $I_{Cir.swell}$.

The properties of $I_{Cl.ir}$ described in the present study are consistent with currents generated by ClC-2 channels, including the cardiac form ClC-2α, when expressed in *Xenopus* oocytes[19,20] or mammalian cell lines.[46] Furthermore, we found that ClC-2 transcripts were present in both atrial and ventricular tissue and isolated single myocytes of mouse and guinea pig heart. Our results, therefore, provide the first compelling evidence for the functional expression of a novel Cl^--dependent inward rectifier that may be encoded by the ClC-2 gene in native mammalian cardiac cells and support a potentially important role of ClC-2 in cardiac function.

Possible Functional Role and Significance. In general, the physiological role of ClC-2 channels remains uncertain because most studies have been carried out only on recombinant ClC-2 channels.[9,18–20] The volume sensitivity of the channel suggests some role in cell volume regulation. Volume regulatory mechanisms are critical in maintaining structural integrity and proper cellular functions of living cells. Cardiac myocytes, like other mammalian cells, are able to use a variety of mechanisms to precisely maintain their size in the face of osmotic perturbations. It is now well known that the outwardly rectifying $I_{Cl.vol}$ plays a role in volume regulation of cardiac and noncardiac cells.[52–56] However, ClC-2 channels differ from the typical $I_{Cl.vol}$ investigated in cardiac and noncardiac cells in terms of their anion selectivity, pharmacology, and rectification properties.[5,9,10,18–20] In fact, $I_{Cl.vol}$ in heart, and possibly in other tissues as well, may be encoded by ClC-3.[10] Furukawa et al[19] recently found that ClC-2G (ClC-2α), when expressed in *Xenopus* oocytes, contributes to volume regulation in the face of osmotic perturbations. However, in human intestinal T84 cells, it was found that only the TMX-sensitive outwardly rectifying $I_{Cl.vol}$ was involved in volume regulation but not the Cd^{2+}-sensitive inwardly rectifying ClC-2–like Cl^- current.[33] How ClC-2 channels are involved in the volume regulation of cardiac cells and their relationship to ClC-3 and other Cl^- channels involved in volume regulation, such as CFTR channels,[57] needs further investigation.

Considering the well-established physiological significance of cationic inward rectifiers like Kir[58,59] and I_f[21] in the regulation of resting membrane potential, action potential duration, and pacemaker activity, it is conceivable that

anionic inward rectifiers may also play a significant role in cardiac electrical activity. Under physiological conditions, the Cl^- equilibrium potential (E_{Cl}) is more positive (−65 to −30 mV) than the resting membrane potential.[60–62] At negative membrane potentials, activation of ClC-2 channels would promote Cl^- efflux and the generation of significant inward current because of their inwardly rectifying properties, thus potentially contributing to the regulation of resting membrane potentials. In the present study, given that functional $I_{Cl,ir}$ could only be demonstrated in a small percentage of isolated mouse and guinea pig atrial and ventricular myocytes, it may be that the physiological significance of these channels in these cell types only becomes prominent under some pathological conditions (ischemia or hypoxia).[63] It is possible that $I_{Cl,ir}$ may normally play a much more prominent role in pacemaker cells in the sinoatrial or atrioventricular nodal regions of the heart, in a manner analogous to the physiological role of I_f channels and their known tissue distribution pattern in heart.[22,64,65] Future studies should be performed to examine functional and molecular expression of ClC-2 in nodal regions of the heart. Finally, additional functional significance of ClC-2 expression in some types of cardiac cells may be related to the formation of heteromultimers with other ClC Cl^- channel family subunits[66] to form unique, yet to be characterized, Cl^- channel subtypes.

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A Novel Anionic Inward Rectifier in Native Cardiac Myocytes

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