Molecular distribution of volume-regulated chloride channels (ClC-2 and ClC-3) in cardiac tissues

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Britton, Fiona C., William J. Hatton, Charles F. Rossow, Dayue Duan, Joseph R. Hume, and Burton Horowitz. Molecular distribution of volume-regulated chloride channels (ClC-2 and ClC-3) in cardiac tissues. Am J Physiol Heart Circ Physiol 279: H2225–H2233, 2000.—The molecular identification of cardiac chloride channels has provided probes to investigate their distribution and abundance in heart. In this study, the molecular expression and distribution of volume-regulated chloride channels ClC-2 and ClC-3 in cardiac tissues were analyzed and quantified. Total RNA was isolated from atria and ventricles of several species (dog, guinea pig, and rat) and subjected to a quantitative RT-PCR strategy. ClC-2 and ClC-3 mRNA expression were calculated relative to β-actin expression within these same tissues. The transcriptional levels of ClC-3 mRNA were between 0.04–0.08% and 0.03–0.18% of β-actin expression in atria and ventricles, respectively (n = 3 for each tissue). The levels of ClC-2 in both atria and ventricles were significantly less than those measured for ClC-3 (n = 3; P < 0.05). ClC-2 mRNA levels were between 0.04–0.08% and 0.03–0.18% of β-actin expression in atria and ventricles, respectively (n = 3 for each tissue). Immunoblots of atrial and ventricular wall protein extracts demonstrated ClC-2- and ClC-3-specific immunoreactivity at 97 and 85 kDa, respectively. Immunohistochemical localization in guinea pig cardiac muscle demonstrates a ubiquitous distribution of ClC-2 and ClC-3 channels in the atrial and ventricular wall. Confocal analysis detected colocalization of ClC-2 and ClC-3 in sarcolemmal membranes and distinct ClC-3 immunoreactivity in cytoplasmic regions. The molecular expression of ClC-2 and ClC-3 in cardiac tissue is consistent with the proposed role of these chloride channels in the regulation of cardiac cell volume and the modulation of cardiac electrical activity.

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With the molecular identification of CIC-2 and CIC-3 as volume-regulated Cl⁻ currents in cardiac myocytes, we now have molecular tools to determine the distribution and abundance of these channels in cardiac tissues. In this study, we used quantitative reverse transcription polymerase chain reaction (RT-PCR) in combination with immunohistochemistry and Western blotting to examine the RNA expression and protein distribution of CIC-2 and CIC-3 channels in cardiac atrial and ventricular tissues from various species. Our results indicate that both CIC-2 and CIC-3 are colocalized in the sarcolemmal membrane of both atrial and ventricular myocytes, consistent with their functional roles as sarcolemmal chloride channels that contribute to the regulation of electrical activity and other cellular functions. A preliminary report of these results has been published (3).

MATERIALS AND METHODS

RNA isolation and cDNA synthesis. Total RNA was isolated from atrial and ventricular tissues using the Trizol reagent (Life Technologies, Gaithersburg, MD), and following the manufacturer's instructions. Total RNA was incubated with RNase-free DNase (Promega, Madison, WI) for 20 min at 25°C, followed by heat inactivation at 90°C. Total RNA (1 μg) was reverse transcribed with 200 units of Superscript II reverse transcriptase (Life Technologies) in a 20-μL reaction containing 25 ng of oligo(dT)₁₂₋₁₈ primer, 500 μM each dNTP, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol (DTT).

Competitive quantitative RT-PCR. Quantitative RT-PCR was performed using the PCR MIMIC construction kit (Clontech, Palo Alto, CA). MIMIC DNA fragments were constructed so that sequences specific for the target gene (CIC-2, CIC-3, or β-actin) were incorporated into the ends of each MIMIC construct. Competitive PCR was then performed with a pair of gene-specific primers that amplify efficiently both the MIMIC DNA and the target cDNA. Known concentrations of MIMIC DNA (10-fold serial dilutions) were titrated with constant amounts of target cDNA. PCR was performed in 25-μL reactions containing Taq buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, and 0.1% Triton X-100), 250 μM each dNTP, 20 μM each primer, 2.5 μL of cDNA, and 1 U of Taq polymerase (Promega). Amplifications were performed in a GeneAmp 2400 thermal cycler (Perkin Elmer, Hercules, CA) for 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. Gene-specific primers were CIC-2 (GenBank accession no. X64139; forward 2,515–2,546 bp, reverse 2,803–2,824 bp); CIC-3 (GenBank accession no. U83464; forward 1,891–1,911 bp, reverse 2,130–2,150 bp); and β-actin (GenBank accession no. V01217; forward 2,282–2,400 bp, reverse 3,071–3,090 bp). RT-PCR products were resolved on 2% agarose gels alongside a molecular weight marker. The amount of target cDNA was determined from the MIMIC dilution in which MIMIC and target cDNA were equal. Gels were analyzed with Molecular Analyst software (Bio-Rad, Foster City, CA). Data were expressed as percentages of β-actin gene expression within the same tissue (n = 3 for each species). To confirm the correct amplification of either CIC-2 or CIC-3, PCR-generated fragments were sequenced with the use of the ABI Prism cycle sequencing kit (Perkin Elmer) and analyzed on a Genetic Analyzer (model 310; Perkin Elmer). There are a number of factors that can affect the relative efficiency of the different PCR reactions, such as primer specificity and unequal amplification of the standard and the target gene (17). However, this study controlled for primer specificity by using primers that had 100% cross-species homology. The amplicon generated from each species used was completely sequenced, and quantitative PCR primers were designed in a nested fashion such that there were no species differences in CIC-2 or CIC-3 sequence in the primer annealing regions.

Northern blot analysis. Total RNA (10–20 μg) from atrial and ventricular tissue was size fractionated on 1% agarose–formaldehyde gels alongside a 0.24- to 9.5-kb RNA ladder (Life Technologies) and transferred to nylon filters. Filters were baked and prehybridized in 50% formamide, 5× SSC (standard sodium citrate), 50 mM sodium phosphate, 5× Denhardt's solution, 50 μg/ml sonicated salmon sperm DNA, 0.1% SDS, and 10% dextran sulfate at 42°C overnight. A 420-bp CIC-2 cDNA fragment and a 500-bp CIC-3 cDNA fragment were radiolabeled with ³²PdCTP by random priming (15). The filters were washed at high stringency (3 times in 2× SSC at room temperature for 5 min and then twice in 0.2× SSC/0.1% SDS at 65°C for 30 min) to ensure specificity of labeling. Filters were exposed to film, and autoradiography was performed using a phosphorimager (Bio-Rad).

Western blot analysis. Crude protein lysate was prepared from atrial and ventricular tissue. Tissue (20–50 mg) was homogenized in buffer containing 10 mM HEPES, pH 7.4, 10% sucrose, and a cocktail of protease inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonfyl fluoride, 5 mM NaF, 1 μM leupeptin, 10 mM EGTA, and 1 mM Na₂EDTA). The supernatant was cleared from cellular debris by centrifugation at 2,500 g for 15 min at 4°C. Protein concentration was assayed by the bicinchoninic acid method (44) with bovine serum albumin (BSA) as a standard. Protein (50–70 μg) from each tissue in 1× SDS buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM DTT, and 0.03% bromophenol blue) was size fractionated on an 8% SDS polyacrylamide gel at 200 V for 50 min in electrode buffer (250 mM Tris, 2 M glycine, and 35 mM SDS). A broad-range protein standard marker (Bio-Rad) was included. Proteins were transferred onto nitrocellulose with the use of the Genie Electroblotter (IDEA Scientific) at 24 V at 4°C for 1 h in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. The blot was blocked in 5% nonfat milk in TNT buffer (100 mM Tris, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The blot was incubated at 4°C overnight with either CIC-2 or CIC-3 affinity-purified monoclonal antisera (1:200 dilution in TNT/0.1% gelatin; Alomone Labs, Jerusalem, Israel). Incubation was followed by washing in 10 ml of TNT (3 times for 5 min) and incubation for 90 min with anti-rabbit IgG alkaline phosphatase conjugate (1:7,500 dilution in TNT/0.1% gelatin; Promega). The blot was washed in TNT (3 times for 15 min), and specific CIC-2 or CIC-3 immunoreactivity was detected colorimetrically with the alkaline phosphatase substrates (50 mg/ml nitroblue tetrazolium and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine) in 100 mM Tris, 100 mM NaCl, and 5 mM MgCl₂, pH 9.5. As a negative control, both antibodies were preabsorbed with the respective antigen-glutathione S-transferase (GST) fusion peptide used to generate that antibody.

Immunohistochemistry. Guinea pig hearts wereperfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), bisected coronally, further fixed with paraformaldehyde for 30 min, washed (4 times for 15 min) in PBS, and cryoprotected in a graded series of sucrose solutions (5, 10, 15, and 20% wt/vol made up in PBS, 1 h each). Both halves of the heart were then embedded in Tissue Tek embedding medium (Miles, IL) and 20% sucrose in PBS (1:2 vol/vol) and
rapidly frozen in isopentane precooled in liquid nitrogen. Cryosections were cut with a Leica CM 3500 cryostat at a thickness of 10 μm and were collected on Vectabond (Vector Laboratories, Burlingame, CA)-coated microscope slides. Sections were initially blocked with 10% BSA for 1 h and then incubated overnight with anti-ClC-2 or anti-ClC-3 antibodies were raised in rabbit (Alomone Labs, Jerusalem, Israel), washed with PBS, and incubated for 1 h with fluorescein isothiocyanate (FITC) or Texas Red anti-rabbit secondary antibodies at 5 μg/ml (Vector Laboratories), washed with PBS (3 times for 10 min), and mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA). Colocalization studies were performed by first incubating sections with ClC-3 antibody and Texas Red anti-rabbit secondary antibody. Sections were then washed (4 times for 15 min) with PBS, incubated with ClC-2 antibody conjugated with FITC by incubating sections with biotinylated anti-rabbit secondary antibody at 5 μg/ml for 1 h, washed (2 times for 15 min) with PBS, incubated with FITC-avidin D at 5 μg/ml for 1 h (Vector Laboratories), washed (3 times for 10 min) with PBS, and then mounted. Sections incubated 1) without primary antibodies or 2) with preabsorbed primary antibodies (absorbed for 1 h with appropriate antigen) were used as negative controls. Sections were examined with the use of a Bio-Rad MRC 600 confocal microscope with excitation wavelengths appropriate for FITC and Texas Red. Confocal micrographs were obtained from digital composites of two-serial scans of 10 optical sections (Z) through a depth of 10 μm (10 × 1 μm), Z series were constructed with Bio-Rad Comos software, and final images were prepared using Adobe Photoshop software.

Statistical analysis. Experiments utilizing PCR to determine ClC-2 and ClC-3 expression were performed on tissue isolated from at least three different animals. For quantitative RT-PCR the concentration of the target DNA was normalized to β-actin expression. Data are expressed as means ± SE, n is the number of animals. One-way ANOVA was used to compare gene expression between the groups, and a Student-Newman-Keuls post hoc test was then used to identify differences among the groups. A two-tailed probability (P) of <0.05 indicates statistical significance.

RESULTS

Molecular expression of ClC-2 and ClC-3 transcripts in cardiac muscle. Northern blot analysis (Fig. 1, A and B) indicates that both ClC-2 and ClC-3 are expressed in the atria and ventricles of guinea pigs, dogs, and rats. The ClC-2 transcript yielded a band at 3.3 kb in all the species examined, which is similar to the transcript size reported by others (6, 48). The size of the ClC-3 transcript obtained by Northern analysis was 3.4 kb in guinea pig and rat and 4.0 kb in dog. Transcript sizes of 3.4 and 5.0 kb have been obtained for ClC-3 RNA expression in cardiac myocytes (13) and other tissues (32). The additional transcript size of 5.0 kb observed in our previous study (13) may be due to the use of poly(A⁺) RNA.

Quantitative analysis of ClC-2 and ClC-3 transcripts in cardiac muscle. Quantitative RT-PCR was used to quantify the expression of ClC-2 and ClC-3 transcripts relative to a housekeeping gene (β-actin). Specific primers were designed for ClC-2 and ClC-3 so that they did not cross hybridize with other members of the ClC family. Qualitative RT-PCR indicated that ClC-2 and ClC-3 mRNA were present in cardiac tissue. The ClC-2 and ClC-3 PCR generated fragments (301 and 276 bp, respectively) were sequenced to confirm the correct amplification of either transcript. We used the competitive “mimic” approach of quantitative RT-PCR to determine the relative amounts of ClC-2 and ClC-3 transcripts in RNA isolated from atrial and ventricular tissues. In competitive RT-PCR, a dilution series of the standard RNA is coamplified with equal amounts of total RNA (and therefore equal amounts of amounts of the native gene). The MIMIC standard competes with the native gene for primers and enzyme, thus reducing the signal for the native gene when the MIMIC standard is in excess. As the amount of native gene increases, the MIMIC standard signal decreases. A representative gel used for digital analysis and comparison of ClC-2- and ClC-3-specific amplification with “mimic DNA” amplification is shown in Figs. 2A and 3A, respectively. As the concentration of MIMIC was reduced from 100 to 0.1 amol/μl, the MIMIC band reduced in density, whereas the ClC-2 and ClC-3 bands increased in density. The amount of target cDNA was determined from the MIMIC dilution in which MIMIC and target cDNA were equal. Molecular Analyst software (Bio-Rad) was then used to accurately determine the ratio of density between the fluorescence of target cDNA and MIMIC bands. This ratio is included in the calculation of the concentration of target gene. The RT-PCR experiments were quantified by comparing ClC-2 and ClC-3 expression to the amount of β-actin gene expression (Figs. 2B and 3B, respectively). In the guinea pig, rat, and dog, quantitative RT-PCR experiments revealed significantly greater amounts of ClC-3 than ClC-2 transcripts in both atrial and ventricular tissue (P < 0.05; n = 3 for each species examined).
normalized values for ClC-3 expression are much larger for the tissues examined (10- to 40-fold) compared with those of ClC-2 expression within the same tissues. ClC-3 expression was 1.8 and 10.2% of β-actin in atria and between 3.4 and 8.6% of β-actin in ventricles, whereas ClC-2 expression was 0.04–0.08% and 0.03–0.18% of β-actin expression in atrial and ventricular tissue, respectively. No significant difference in ClC-3 expression in atria and ventricles was observed for any of the species examined. ClC-2 expression was significantly higher in the ventricles than atria of rats (P < 0.01; n = 3) and significantly higher in the atria than ventricles of canine tissue (P < 0.01; n = 3). ClC-2 expression appears higher in the ventricles than atria of guinea pigs, but this finding was not statistically significant (P > 0.05; n = 3). Increasing our n values may have reduced the variability, and the difference in ClC-2 expression may be statistically significant.

Western analysis of ClC-2 and ClC-3 protein in cardiac muscle. Expression of ClC-2 and ClC-3 polypeptides in atrial and ventricular tissue was detected by immunoblotting with the use of affinity-purified polyclonal antibodies generated against GST fusion peptides corresponding to amino acid residues 888–906 of rat ClC-2 (Fig. 4A) and residues 592–661 of rat ClC-3 (Fig. 4B). ClC-2 and ClC-3 antibody specificity was confirmed in Western blots with the use of antibody that had been preabsorbed with purified CIC-2 or CIC-3 antigen that was used to generate the antibodies. The preabsorbed antibodies did not react with proteins isolated from the cardiac tissue. The CIC-2 antisera recognized a single band corresponding to a polypeptide of ~97 kDa, the expected size of the protein predicted from cDNA sequence analysis (48). CIC-3 protein migrated with a molecular mass of 85 kDa. This molecular mass is close to that predicted from the sequence of CIC-3 (32). Additional CIC-3-like immunoreactivity was observed in these homogenates at 65 and 70 kDa (Fig. 4B). These smaller molecular mass bands were also eliminated with the preabsorbed antibody and may represent different glycosylated CIC-3 forms (42) or CIC-3 proteolysis products. CIC-3 has considerable sequence homology with the chloride channels CIC-4 and CIC-5. The epitope fragment used to generate the CIC-3 polyclonal antibody (70 amino acids in the COOH terminus) has considerable homology with CIC-4 and CIC-5 proteins (46/70 and 49/70 identities in the carboxy terminus of rat CIC-4 and CIC-5, respectively). There is the possibility that there may be some degree of cross-reactivity with these related proteins in cardiac tissue. However, Schmieder et al. (42) observed no cross-reaction of the same CIC-3 antisera in immunoblots of *Xenopus laevis* oocyte membrane preparations expressing exogenous rat CIC-4 or rat CIC-5.

Immunohistochemical localization of ClC-2 and ClC-3 in cardiac muscle. The localization of ClC-2 and ClC-3 channels in cardiac tissue was determined im-

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Fig. 2. Quantitative RT-PCR of ClC-2 gene expression in cardiac muscle. A: representative gel of quantitative RT-PCR for ClC-2 in canine ventricle. Known concentrations of MIMIC DNA (0.01–100 amol/μl) were coamplified with unknown concentrations of target cDNA. Competitive RT-PCR products were resolved on 2% agarose gels alongside a 100-bp marker. The MIMIC DNA band is visible at 574 bp and the ClC-2 band at 309 bp. The amount of ClC-2 cDNA can be determined from the MIMIC dilution in which the ClC-2 and ClC-3 bands appear equal. Quantitative RT-PCR for β-actin expression was also performed from the same cDNA.

Fig. 3. Quantitative RT-PCR of ClC-3 gene expression in cardiac muscle. A: representative gel of quantitative RT-PCR for ClC-3 in canine ventricle. Known concentrations of MIMIC DNA (0.1–100 amol/μl) were coamplified with unknown concentrations of cDNA. Competitive RT-PCR products were resolved on 2% agarose gels alongside a 100-bp marker. The MIMIC DNA band is visible at 600 bp and the ClC-3 band at 300 bp. The amount of target DNA can be determined from the MIMIC dilution in which the MIMIC and ClC-3 bands appear equal. Quantitative RT-PCR for β-actin expression was also performed from the same cDNA. B: concentrations of ClC-3 gene expression in various cardiac tissue were calculated and reported relative to β-actin expression within the same tissue (n = 3 for each species).
at the sarcolemmal membrane, where it is colocalized (Red). ClC-2 appears to be more strongly expressed than ClC-3 as determined by immunohistochemistry on guinea pig heart left ventricular wall that was sectioned both longitudinally (Fig. 6, A–C) and transversely (Fig. 6, D–F). Confocal immunofluorescence shows that individual ventricular myocytes are immunoreactive for both ClC-2 (FITC green) and ClC-3 (Texas Red). ClC-2 appears to be more strongly expressed at the sarcolemmal membrane, where it is colocalized (yellow) with ClC-3 (Fig. 6, C and F). Immunoreactivity was not observed when sections were incubated without primary antibodies or with antibodies preabsorbed with purified ClC-2 or ClC-3 fusion protein.

**DISCUSSION**

Volume-regulated Cl\(^{-}\) currents are present in numerous cell types, including cardiac myocytes, and play important roles in the control of cell volume, pH, and membrane potential (16, 26, 27). However, the molecular identity of these anion channels has been a recent matter of debate (7, 39, 47). Many candidates have been proposed for the chloride conductance path that mediates regulatory volume decrease (RVD) (38). The molecular candidates include P-glycoprotein (pGp), the product of the multidrug resistance gene (51), and pICln (41), which, when expressed in fibroblasts, has been reported to be responsible for a Cl\(^{-}\) conductance activated by hypotonic solutions (24). Although it now appears that pGp and pICln may not actually be anion channels, their involvement in cell swelling raises the possibility that these proteins may modulate the volume-regulated Cl\(^{-}\) conductance in heart tissue.

ClC-2 and ClC-3, which certainly encode chloride channel proteins and are members of the ClC family of voltage-gated chloride channels, have been reported to be sensitive to changes in cell volume (13, 23). In addition, the ubiquitous expression of ClC-2 and ClC-3 in many cell types (32, 48) implies that ClC-2 and ClC-3 may play a role in a universal and necessary function in many types of cells.

ClC-2 and ClC-3 as candidates for volume regulation in cardiac myocytes. The functional expression of guinea pig cardiac ClC-3 (gpClC-3) in mammalian NIH/3T3 cells (9, 13) results in a large basally active Cl\(^{-}\) conductance, which is strongly modulated by cell volume and exhibits many of the same biophysical and pharmacological properties as the native Cl\(_{\text{Cl,vol}}\) present in cardiac myocytes (11, 53). Thus ClC-3 may be the gene responsible for Cl\(_{\text{Cl,vol}}\) found in cardiac myocytes of a variety of species (29). However, the identification of ClC-3 as the molecular counterpart responsible for native Cl\(_{\text{Cl,vol}}\) in some cells has certainly not been exempt from controversy (see Refs. 30, 39, and 50 for review).

ClC-2 currents are characterized by having inward rectification, time-dependent activation at hyperpolarizing voltages and a halide selectivity of Cl\(^{-}\) > I\(^{-}\). ClC-2 channels expressed in *Xenopus* oocytes have been shown to increase with large hyperpolarizing voltages as well as extracellular hypotonicity (23). A hyperpolarization-activated ClC-2-like current that is also modulated by cell volume has been characterized in the human T\(_{84}\) adenocarcinoma cell line (2, 18) and in other noncardiac tissues (4, 8, 40). Recently, our group has demonstrated that similar volume-regulated inwardly rectifying currents (Cl\(_{\text{Cl,irr}}\)) can be recorded from native mouse and guinea pig cardiac myocytes (14). We have now found that ClC-2 is ubiquitously expressed in atria and in the ventricular wall including the epicardium and endocardium.
Localization of ClC-2 and ClC-3 channels to myocyte membranes. ClC-2-specific antibody predominantly stains cardiomyocyte sarcolemmal membranes. ClC-3-specific antibody stains both sarcolemmal and what appears to be intracellular membranes, which may indicate some cytoplasmic staining. However, it is possible that this apparent cytoplasmic staining may actually be due to the presence of ClC-3 in T-tubular membranes, which are known to have a complex reticular organization in cardiac myocytes (45). The presence or absence of ClC-3 in T tubules is important to investigate and may have implications for ClC-3 function. A followup study is required to evaluate the localization of ClC-3 in sarcolemmal and T-tubular membranes with the use of dual labeling with specific markers (e.g., di-8-ANEPPS; Ref. 43) on isolated atrial and ventricular cells. It is possible that the difference in staining pattern between ClC-2 and ClC-3 reflects functional differences of the two channels. However, the observed colocalization of the two channels at the sarcolemmal membrane suggests a role for both proteins as sarcolemmal chloride channels in myocytes and presents the intriguing possibility of ClC-2/ClC-3 heterodimer formation. The latter needs to be investigated by in vitro experiments.

CIC-2 versus CIC-3: a role in myocyte volume regulation. The role of CIC-2 in volume regulation may well depend on its expression level relative to other swelling-activated chloride channels. T84 cells possess at least two distinct swelling-activated chloride conductance paths, one mediated by a ClC-2-like current (i.e., inwardly rectifying and Cd$^{2+}$-sensitive, tamoxifen insensitive), $I_{\text{Cl,ir}}$, and the other by a swelling-activated, tamoxifen-sensitive outwardly rectifying chloride current, $I_{\text{Cl,vol}}$, possibly encoded by ClC-3 (2). Bond and colleagues (2) suggested that $I_{\text{Cl,vol}}$ rather than $I_{\text{Cl,ir}}$ mediates RVD in T84 cells on the basis of the sensitivity of RVD to specific inhibitors of $I_{\text{Cl,vol}}$ or $I_{\text{Cl,ir}}$. Possibly, CIC-2 does not contribute significantly to RVD in T84 cells because it is not expressed at the same levels as the channel that mediates $I_{\text{Cl,vol}}$.

A similar conclusion can be made in cardiac myocytes. In a recent study, we observed that only a small population of mouse and guinea pig atrial and ventricular myocytes appear to exhibit hyperpolarization-activated $I_{\text{Cl,ir}}$ (14). In this study, we used a competitive RT-PCR approach to quantify the level of ClC-2 and ClC-3 mRNA transcripts in cardiac tissue. Quantitatively, at the transcriptional level, we observed significantly lower levels of ClC-2 mRNA transcript expres-
sion compared with ClC-3 expression in both atrial and ventricular myocytes. This is consistent with a higher percentage of cells exhibiting functional outwardly rectifying ClC-2-like currents compared with inwardly rectifying ClC-2-like currents. Although this quantitative difference is not obvious in immunoblots or immunohistochemistry, these latter approaches are not quantitative in nature and may thus explain the apparent discrepancy between functional swelling-activated chloride channels and ubiquitous molecular ClC-2 expression. When the quantitative RT-PCR data are considered, it must be kept in mind that RNA was extracted from cardiac tissue rather than isolated myocytes, and thus the levels of ClC-2 and ClC-3 transcripts are from a mixed population of cells (see e.g., Ref. 33). Also the amount of mRNA detected by quantitative RT-PCR does not necessarily reflect a corresponding or equal measure of functional ClC-2 or ClC-3 protein. Of interest is the study by Wong and co-workers (55), who measured the density of ClC-3 mRNA levels across the left ventricular wall of rabbit heart by in situ hybridization and who also measured the corresponding current density of swelling-activated chloride channels in myocytes isolated from various regions of the ventricular wall. They found that, although there was uniform expression of ClC-3 mRNA across the ventricular wall, the whole cell slope conductance of swelling-activated chloride channel activity was higher in myocytes isolated from the subepicardium than in myocytes isolated from the midmyocardium or subendocardium. They concluded that the control of gene expression might be less important for regulating the distribution of functional swelling-activated chloride channels in the heart.

Another possible explanation for the discrepancy between the apparent ubiquitous ClC-2 expression and the small population of mouse and guinea pig atrial and ventricular myocytes exhibiting a hyperpolarization activated $I_{\text{Cl,ir}}$ could be that many ClC-2 proteins may form heterodimeric channels with other ClC proteins (perhaps ClC-3) (35), resulting in channels with characteristics different from ClC-2 homodimers. It is also possible that ClC-2 is regulated in a way that renders the channel silent under basal conditions. Both of these possibilities are being studied.

In summary, we have demonstrated the molecular expression of two volume-regulated chloride channels in cardiac myocytes from several species. We have shown that ClC-3 transcriptional expression predominates in all species relative to a housekeeping gene. Finally, we have examined the cellular and subcellular localization of these channels in cardiac tissues and myocytes. Our findings indicate that both ClC-2 and ClC-3 are colocalized in the sarcolemmal membrane of both atrial and ventricular myocytes. This is consistent with their functional roles as sarcolemmal chloride channels that may regulate cardiac cell volume and electrical activity.

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