K⁺-Cl⁻ cotransporter-2 KCC2 in chicken cardiomyocytes

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Antrobus SP, Lytle C, Payne JA. K⁺-Cl⁻ cotransporter-2 KCC2 in chicken cardiomyocytes. Am J Physiol Cell Physiol 303: C1180–C1191, 2012. First published October 3, 2012; doi:10.1152/ajpcell.00274.2012.—Using antibodies prepared against a unique region (exon 22–24) of rat K⁺-Cl⁻ cotransporter-2 (KCC2), we confirmed that the ~140-kDa KCC2 protein is exclusively expressed in rat brain, but in chicken, we observed strong reactivity not only with the ~140-kDa KCC2 protein in brain but also a slightly larger ~145-kDa protein in heart. In silico analysis showed that while exon 22 of KCC2 is unique to this isoform in therian mammals, it is retained in KCC2’s closest paralog, KCC4, of lower vertebrates, including chicken. To eliminate potential cross-reactivity with chicken KCC4, the antibodies were preadsorbed with blocking peptides prepared over the only two regions showing significant sequence identity to chicken KCC4. This completely eliminated antibody recognition of exogenously expressed chicken KCC4 but not of the ~145-kDa protein in chicken heart, indicating that chicken heart expresses KCC2. Real-time PCR confirmed robust KCC2 transcript expression in both chicken brain and heart. Chicken heart expressed predominantly the longer KCC2a splice variant consistent with the larger ~145-kDa protein in chicken heart. Immunofluorescence microscopy revealed prominent plasma membrane KCC2 labeling in chicken ventricular cardiomyocytes. We hypothesize that KCC2 is an important Cl⁻ extrusion pathway in avian cardiomyocytes that counters channel-mediated Cl⁻ loading during high heart rates with β-adrenergic stimulation. While KCC2 is absent from mammalian cardiomyocytes, understanding the role that the other KCC isoforms play in Cl⁻ homeostasis of these cells represents a nascent area of research.

cell volume regulation following hyposmotic swelling (8, 10, 39). In addition to cell volume regulation, key functional roles have been described for the KCCs in epithelia and neurons. For example, in acid-secreting epithelial cells (e.g., renal distal nephron α-intercalated cells), KCC4 appears to operate as a basolateral Cl⁻ efflux pathway that helps recycle Cl⁻ entering via anion exchange (7). A more novel function is evident in the central nervous system (CNS), where the developmental up-regulation of KCC2 is associated with maturation of postsynaptic GABAergic inhibition in central neurons (38). That is, KCC2 is largely responsible for lowering intracellular [Cl⁻] during neuronal maturation so that the Cl⁻ reversal potential (ECl) is more negative than the resting membrane potential (22, 32, 38). Hence, KCC2 is critical for the development of fast hyperpolarizing synaptic inhibition mediated by GABA_A receptors. The KCC2 protein also appears to play a morphogenetic role, independent of its ion transport activity, in dendritic spine formation (16, 20, 28). Since KCC2 has been implicated in the development of both inhibitory and excitatory neurotransmission, it is emerging as a key “synchronizing factor” in the developing nervous system (28).

KCC2 stands out among the KCC isoforms not only in terms of its unique operation in neurons but also in terms of its structure. KCC2 contains an exon (exon 22 encoding 41 amino acids) that has long been believed to be unique to this isoform of the KCCs. This exon represents a highly charged intracellular region of the protein with a number of serine and threonine residues, leading us to propose the likelihood of it serving as a unique regulatory region (33). Indeed, we have identified a protein kinase-C phosphorylation site encoded by this exon (Ser-940 of rat KCC2) that appears to be involved in modulating the membrane trafficking of KCC2 (27). To better understand the functional role of the unique carboxy-terminal region of KCC2 encoded by exon 22, we undertook an in silico analysis of this region. During our analysis, we discovered that while the sequence of exon 22 is unique to KCC2 in therian mammals (i.e., human, rat, and mouse), it is nonetheless present in KCC4 of lower vertebrates, including fish, birds, and a prototherian mammal (i.e., platypus). This led us to the discovery that a set of KCC2 antibodies that we had previously prepared against a fusion protein encoding most of exon 22 of rat KCC2 (46) strongly recognizes a ~145-kDa protein in chicken heart. The biochemical and molecular studies presented in this report demonstrate that a single splice variant of KCC2 (KCC2a) is robustly expressed in the plasma membranes of cardiomyocytes throughout both chicken ventricles. We speculate that K⁺-Cl⁻ cotransport is critical for efficient intracellular Cl⁻ homeostasis especially under conditions of elevated heart rate following β-adrenergic stimulation, when channel-mediated Cl⁻ loading [via cystic fibrosis transmembrane conductance regulator (CFTR) and Ca²⁺-activated Cl⁻ channels] is predicted to be most prominent.

The K⁺-Cl⁻ cotransporter (KCC) is a member of the cation chloride cotransporter (CCC) gene family and mediates the obligatorily coupled, electroneutral movement of K⁺ and Cl⁻ across the plasma membrane. Under normal physiological conditions, KCC functions as a net K⁺-Cl⁻ efflux pathway that uses the favorable K⁺ chemical gradient to drive Cl⁻ out of the cell against its chemical gradient. Before its characterization at the molecular level, KCC was most often studied in single cells such as red cells where it was implicated in the regulation of cell volume following swelling by promoting the efflux of K⁺, Cl⁻, and osmotically obliged water. Few researchers could have predicted the genetic and functional diversity that is now evident among the KCC isoforms. Within the vertebrate CCC family, the KCCs exhibit the greatest genetic diversity with four separate isoforms, KCC1–4. Further genetic diversity of the KCCs is manifest as a result of alternative splicing, including alternate promoters and alternate first exons (30, 41). Studies using KCC1−/− and KCC3−/− mice have provided strong evidence that KCC3 is the primary isoform involved in chloride; homeostasis; KCC2; KCC4; avian; Cl⁻ channels.

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MATERIALS AND METHODS

Selective tissue harvest. Tissues from both rats and chickens were obtained and used according to approved protocols by the Institutional Animal Care and Use Committee at the University of California, Davis. Fertilized chicken eggs, adult chickens, and adult rats were obtained from a local source. Chicken eggs were maintained in a humidified incubator at 37°C, and tissues were isolated before embryonic day 18. Chicken embryos were euthanized by decapitation, and adult chickens and rats were euthanized by CO2 inhalation.

RNA isolation and cDNA synthesis. Tissues were isolated by dissection and immediately homogenized in a guanidinium thiocyanate solution or quickly frozen in liquid nitrogen. Total RNA was isolated from chicken and rat tissues using the guanidinium thiocyanate method (11) and RNeasy Fibrous Tissue Midi kit (Qiagen). Poly(A) counting method and RNase H treatment (Qiagen). Poly(A)+ RNA was isolated using the PolyA Tract system (Promega), following treatment of total RNA with RNase I. Complementary DNA was generated from 100 ng of mRNA or 1 µg total RNA using RETROscript (Ambion) with oligo(dT) primers, random decamers, or gene-specific primers. Oligonucleotide primers used for real-time PCR are listed in Table 1.

Cloning chicken KCC1 (Slc12a4). The full-length sequence of chicken (Gallus gallus) KCC1 mRNA (XM001234004) was identified in the NCBI database. With the use of sequence information from the chicken KCC1 cDNA (XM001234004), three sets of forward and reverse primers were synthesized and used to amplify fragments from double-stranded cDNA isolated from chicken brain or heart (see RNA isolation and cDNA synthesis). Each reaction used a final volume of 50 µl, containing 2.5 µM of the double-stranded chicken brain or heart cDNA, 5 µM of each primer, 50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane, Tris-HCl (pH 8.3, 25°C), 1.5 mM MgCl₂, 0.125 mM dNTP, and 3 U of Taq DNA polymerase (In Vitrogen). Twenty-five cycles of PCR were performed for each reaction, consisting of incubation of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C from the three reactions, three separate PCR products of appropriate size were obtained and subcloned into pCR 2.1 TOPO (Invitrogen). The full-length version of chicken KCC1 was cloned into the expression vector pB20.

Cloning chicken KCC2 (Slc12a5). The predicted sequence of chicken (Gallus gallus) KCC2 mRNA (XM001236721) was identified in the NCBI database. We noted that this sequence correctly encoded a KCC2 mRNA (XM001236721) was identified from NCBI using the BLAST search program (1) and rat KCC2 as the queried sequence. With the use of sequence information from the chicken KCC4 cDNA (NM001006371), four sets of forward and reverse oligonucleotide primers were synthesized and used to amplify fragments from double-stranded cDNA from whole chicken brain or heart (see RNA isolation and cDNA synthesis). Each PCR reaction was performed using similar conditions to those described above for cloning of chicken KCC1. From the three of the reactions, three separate PCR products of appropriate size were obtained. In a fourth reaction, two distinct PCR products were obtained, corresponding to cDNA with (S1) and without (S2) exon 22 (117 bp) from the chicken Slc12a7 (KCC4) gene. All five reaction products were subcloned into pCR 2.1 TOPO (Invitrogen). To follow protein production in expression experiments, we used PCR mutagenesis to add the c-myc epitope (EQKLISEEDL) to the amino terminus of chicken KCC4-S1 and KCC4-S2. The full-length versions of c-myc tagged chicken KCC4-S1 and KCC4-S2 were cloned into the expression vector pB20.

RT-PCR and semi-quantitative real-time PCR. RT-PCR was also used to determine KCC2 expression in various chicken tissues. Primers (S1) and without (S2) exon 22 (117 bp) from the chicken Slc12a7 (KCC4) gene. All five reaction products were subcloned into pCR 2.1 TOPO (Invitrogen). To follow protein production in expression experiments, we used PCR mutagenesis to add the c-myc epitope (EQKLISEEDL) to the amino terminus of chicken KCC4-S1 and KCC4-S2. The full-length versions of c-myc tagged chicken KCC4-S1 and KCC4-S2 were cloned into the expression vector pB20.

Cloning chicken KCC4 (Slc12a7). The full-length predicted sequence of chicken (Gallus gallus) KCC4 protein (XP001233067) was identified from NCBI using the BLAST search program (1) and rat KCC2 as the queried sequence. With the use of sequence information from the chicken KCC4 cDNA (NM01006371), four sets of forward and reverse oligonucleotide primers were synthesized and used to amplify fragments from double-stranded cDNA from whole chicken brain or heart (see RNA isolation and cDNA synthesis). Each PCR reaction was performed using similar conditions to those described above for cloning of chicken KCC1. From the three of the reactions, three separate PCR products of appropriate size were obtained. In a fourth reaction, two distinct PCR products were obtained, corresponding to cDNA with (S1) and without (S2) exon 22 (117 bp) from the chicken Slc12a7 (KCC4) gene. All five reaction products were subcloned into pCR 2.1 TOPO (Invitrogen). To follow protein production in expression experiments, we used PCR mutagenesis to add the c-myc epitope (EQKLISEEDL) to the amino terminus of chicken KCC4-S1 and KCC4-S2. The full-length versions of c-myc tagged chicken KCC4-S1 and KCC4-S2 were cloned into the expression vector pB20.

RT-PCR and semi-quantitative real-time PCR. RT-PCR used to determine KCC2 expression in various chicken tissues were conducted in a volume of 50 µl containing 2 µl of cDNA (see RNA isolation and cDNA synthesis), 5 µl 10× AmpliTaq Gold PCR buffer (Applied Biosystems), 4 µl 10 mM dNTP, 4 µl 50 mM MgCl₂, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 4 µl of 10 µM gene-specific forward and reverse primers, and 26.7 µl diethylpyrocarbonate-treated water. PCR amplification was done using the following program: 95°C for 10 min; 30 cycles of 95°C for 30 s, 60°C for 15 s, and 72°C for 15 s; and final extension at 72°C for 7 min. Ten microliters of the PCR reactions were then run on a 2% agarose gel containing ethidium bromide.

Semi-quantitative real-time-PCR was performed with an Applied Biosystems 7900HT Fast Real-time PCR system with SYBR green fluorescent label. Samples with a final volume of 12 µl contained the following: 0.4 µl of cDNA generated using random decamers, 6 µl 2× Power SYBR Green PCR master mix (Applied Biosystems), 2 µl of each 4.8 µM gene-specific forward and reverse primers (see Table 1), and 1.6 µl diethylpyrocarbonate-treated water. Samples were run in triplicate in 384-well plates. Cycling parameters were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min. After the PCR amplification, a melting curve was created for each sample to verify the specificity of the reactions. All data were compared with those from a housekeeping gene (GAPDH). The crossing threshold (Ct) for each sample was calculated using the Sequence Detection System (SDS) Software v2.3. Outlier values with a difference between Ct and Ct-mean >0.5 were excluded from further data analysis. The kinetic PCR efficiency correction value (E) for each tissue and primer set was determined by completing real-time PCR with serial dilutions of cDNA over a range of 2 µl to 2 × 10⁻⁷ µl. By plotting the corresponding Ct values for each dilution, calculating the slope of the best fit line, we determined E from the equation, E = dilution factor⁻¹/slope. The relative mean normalized expression levels of transcript were determined using the following equation: (Eref/Ct)ref/(Ethawt/Ct)target with GAPDH used as the reference.

Antibody production and purification. We have previously described the production and purification of a fusion protein (termed Table 1. Combinations of gene-specific primers for semi-quantitative real-time PCR

<table>
<thead>
<tr>
<th>Chicken</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>KCC1</td>
<td>TCCCATAGTGAGCAGACTTTG</td>
<td>ACTCTTCCAGACCTGATG</td>
</tr>
<tr>
<td>KCC2</td>
<td>GAGTCGCCTCTCTGCTCAAG</td>
<td>AAAGCTTGTAGCTTCTCAG</td>
</tr>
<tr>
<td>KCCa</td>
<td>GAAGACGCCGCCCTGAAC</td>
<td>TGCCAGATCTCAGCTTGA</td>
</tr>
<tr>
<td>KCCb</td>
<td>AACCTGAGAGAGCTTCCG</td>
<td>GGCGTGCTCCATCCATCC</td>
</tr>
<tr>
<td>KCC4</td>
<td>GCTCTCTGATAGCTTGGCC</td>
<td>CGGATGCTGTCCCTACATTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTTCGCGTGCCGAACCCCC</td>
<td>GGCCAGACAGGAGGCTTCC</td>
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B22) along with the generation and characterization of rabbit polyclonal antibodies (rb-B22-KCC2) against this purified fusion protein (46). Antisera to B22 were generated in guinea pigs (gp-B22-KCC2) by the Polyclonal Antibody Production Program (Comparative Pathology Lab Services of the School of Veterinary Medicine, University of California, Davis). Both rb-B22-KCC2 and gp-B22-KCC2 were purified by affinity chromatography using the B22 fusion protein coupled to agarose beads using previously published methods (46).

Protein analysis. Methods used for preparing membranes, for deglycosylating membrane protein, and for protein analysis by SDS-PAGE and Western blotting have been previously published (46).

Immunocytochemistry. The polyclonal antibody to human cardiac troponin I (raised to 2 peptides residues 39–52 and 195–209) was kindly provided by Dr. Aldrin Gomes (University of California, Davis) and was made by 21st Century Biochemicals. Mouse monoclonal antibodies to chicken Na,K-ATPase-α-subunit were obtained from the Developmental Studies Hybridoma Bank (6). Cryosections (10 μm) of formalin-fixed adult chicken heart (left ventricle) were treated with 1% SDS in PBS for 5 min to unmask antigenic sites and then incubated sequentially in blocking solution (5% goat serum, 2% BSA, 25 mM glycine, 3 mM EDTA, and 0.1% Tween-20 in PBS, pH 7.4, 1 h), primary antibodies (in blocking solution, overnight), and fluorophore-conjugated secondary antibodies (in PBS plus 0.1% Tween-20), with three rinses between each step. Coverslips were mounted over Gelvatol containing the nuclear stain ToPro-3 (Molecular Probes). Fluorescence images were acquired with a Zeiss LSM-510 confocal microscope through a ×25 water immersion (0.8 NA Plan Neofluar) objective.

Stable expression in HEK-293 cells. The human embryonic kidney cell line HEK-293 was maintained in DMEM (Cellgro) supplemented with 10% FBS, penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified incubator (5% CO₂ at 37°C). For stable cell line expression, constructs were transfected into HEK-293 cells by calcium phosphate precipitation using previously described methods (34). After 3 wk of growth in 900 μg/ml geneticin (GIBCO-BRL), single resistant colonies were amplified and screened by Western blot with a c-myc epitope monoclonal antibody (15) or KCC2 rabbit polyclonal antibodies and by 86Rb influx assay.

RESULTS

KCC2 stands apart from the other three K⁺-Cl⁻ cotransporter isoforms both functionally and structurally. Functionally, KCC2 plays a key role in the regulation of intracellular [Cl⁻] of mature neurons and is instrumental in neuronal development and fast synaptic inhibition. Structurally, the KCC2 gene contains an exon (exon 22) encoding 41 amino acids that has long been believed to be unique to this isoform of the K⁺-Cl⁻ cotransporters (Fig. 1A). To better understand the potential functional role of exon 22, we undertook an in silico analysis of this region of KCC2. During our analysis, we identified a similar exon in KCC4 of lower vertebrates, including chicken (Gallus gallus), finch (Taeniopygia guttata), and teleost fish (Danio rerio; Fig. 1B). Remarkably, KCC4 of
platypus (*Ornithorhynchus anatinus*), a prototherian mammal, also contained an exon similar to that of exon 22 of KCC2.

Analysis of the chicken KCC4 gene (*Slc12a7; Chromosome 2; GeneID: 420797*) revealed the presence of 26 exons. Exons 2–26 of chicken KCC4 share significant identity and alignment with the corresponding exons of KCC2 (*Slc12a5*) of therian mammals (e.g., human, rat, and mouse). This brain finding is reasonable as KCC4 is the closest paralog to KCC2. The lack of identity and alignment of exon 1 between KCC4 of chicken and KCC2 of therian mammals is not surprising given that this is a highly variable region among the KCCs and subject to alternative splicing (i.e., alternate first exons) in KCC2 (41). Analysis of the KCC4 gene of therian mammals showed that while the sequence similar to exon 22 of KCC2 is still identifiable within the intron between the bordering exons, it no longer represents coding sequence. Hence, KCC4 from therian mammals is encoded by only 25 exons (Fig. 2). Sequence analysis of the genomes of numerous vertebrates (amphibians, birds, and mammals) revealed the exon corresponding to exon 22 of KCC2 is absent from vertebrate KCC1 (*Slc12a4*) and KCC3 (*Slc12a6*) genes. While KCC3 was evident in an amphibian (*Xenopus tropicalis*) genome, neither its gene nor transcript was apparent in the Ensembl or NCBI databases for *Xenopus tropicalis* genome, neither its gene nor *Xenopus* (*Ornithorhynchus anatinus*), a prototherian mammal, proved unsuccessful. These findings lead us to conclude that within the vertebrates the KCC isoforms have undergone genetic deletion in the avian vertebrate class. Furthermore, we conclude that exon 22 is not unique to KCC2. While it is absent from KCC1 and KCC3 genes of vertebrates, it is present in KCC4 of a prototherian mammal (platypus) and lower vertebrates, including birds and teleost fish. As we will discuss further below, we also noted that a small 15-bp exon (exon 24 in chicken KCC4) is subject to alternative splicing in avian KCC1, avian KCC4, and mammalian KCC4 (Fig. 2). Taken together, our findings indicate that within the vertebrates the KCC isoforms have undergone significant evolution, exhibiting class-specific exon deletion, alternative splicing of exons, and apparent gene deletion.

Biochemical characterization of K⁺–Cl⁻ cotransport protein in chicken heart. We have previously prepared rabbit polyclonal antibodies (rb-B22-KCC2) against a fusion protein containing most of the sequence encoded by exons 22–24 of rat KCC2 (46). This region of KCC2 was chosen for antibody production because of its highly antigenic nature (i.e., high surface probability) and poor sequence conservation with rat KCC1. The rb-B22-KCC2 antibodies broadly recognize the KCC2 protein in brains of vertebrates from mammals to elasmobranchs (46). In therian mammals, these antibodies have neither shown reactivity with proteins outside the CNS nor shown cross-reactivity with any other KCC isoform. Although these antibodies have been determined to be KCC2-specific in therian mammals, they have not been extensively tested in lower vertebrates. Because exon 22 of KCC2 is absent from the other KCC isoforms and the sequence within exon 23 of KCC2 is poorly conserved among the KCC isoforms, the lack of cross-reactivity of rb-B22-KCC2 with the other KCC isoforms is not surprising (Figs. 1 and 2). Sequence corresponding to the small 15-bp exon 24 of KCC2 is absent from both KCC1 and KCC3 of therian mammals (Fig. 2). While this small exon of KCC4 is often not included in database sequences for KCC4 of therian mammals, we have been able to identify it in the KCC4 gene (*Slc12a7*) of human, mouse, and rat. We suspect that the sporadic appearance of this 15-bp exon in database sequences is due in part to its small size, but more importantly, its inclusion in transcripts probably represents a minor alternatively splice variant of KCC4. This hypothesis is supported by the low percentage of KCC4 ESTs that include this exon. Moreover, we rarely found this exon in clones from RT-PCR performed over this region using gene-specific primers for mouse KCC4 or chicken KCC4 (data not shown).

It is noteworthy that the first 15 amino acids encoded by exon 22 of rat KCC2 and KCC4 of lower vertebrates share remarkably high identity, whereas the remaining residues of the exon are poorly conserved among the vertebrate classes (Fig. 1B). This prompted us to test whether rb-B22-KCC2 might recognize chicken KCC4. Since KCC2 is believed to be...
a neuron-specific isoform in therian mammals and KCC4 is found in multiple mammalian tissues, we performed a Western blot with rb-B22-KCC2 against membranes prepared from multiple tissues of rat and chicken (Fig. 3). As we have shown previously, rb-B22-KCC2 recognized a single ~140-kDa protein in rat brain (46). No protein bands were recognized by rb-B22-KCC2 in any other rat tissue even at 20-fold higher protein loads. In membranes isolated from adult chicken tissues, however, rb-B22-KCC2 strongly recognized a 140- to 145-kDa protein present in brain and heart but not other tissues. We have little doubt that the protein recognized in chicken brain is KCC2. Given that KCC2 is believed to be neuron-specific and that rat KCC2 and chicken KCC4 are identical in sequence over an extended region of exon 22 over which the rb-B22-KCC2 antibodies were prepared (Fig. 1B), we postulated that the protein recognized in chicken heart was KCC4.

Importantly, the protein recognized in chicken heart exhibited a slightly larger mass (2–5 kDa) than that in chicken brain. To evaluate whether this size difference reflected differences in core protein mass as opposed to differential glycosylation, we treated membranes from rat brain, chicken brain, and chicken heart with N-linked sugar groups. We have previously demonstrated that treatment of KCC2 from rat brain with N-glycosidase F reduces its apparent mass to ~124 kDa, which is comparable to that of the predicted core protein (46). A similar size reduction was observed for membranes from both rat brain and chicken brain (Fig. 4). When we treated chicken heart membranes with N-glycosidase F, the band detected by Western blot was reduced in size but still recognizable as slightly larger than that from chicken brain by ~2–5 kDa. These data indicate that the size difference of protein recognized by rb-B22-KCC2 in chicken brain and chicken heart is due to differences in the core protein mass. In comparing the chicken KCC core protein masses, chicken KCC1 is ~1 kDa smaller than KCC2 whereas chicken KCC4 is ~2 kDa larger than KCC2. These findings support our hypothesis that the rb-B22-KCC2 antibodies were cross-reacting with KCC4 in chicken heart. To test this hypothesis, we cloned KCC4 from chicken brain and heart using a PCR strategy along with the known sequence from the NCBI database (see MATERIALS AND METHODS). During the cloning of chicken KCC4, we noted that exon 22 was subject to alternative splicing as we obtained two different sized products in one of our PCR reactions (see MATERIALS AND METHODS), i.e., one with and one without this exon. We termed these alternative splice variants of chicken KCC4, KCC4-S1 (with exon 22), and KCC4-S2 (without exon 22). After cloning both KCC4 variants, expression constructs of each were prepared with a c-myc epitope tag at the amino terminus and then expressed in stable HEK-293 cell lines. As noted above, the small 15-bp
exon 24 was rarely identified in our PCR reactions; hence, our KCC4-S1 and KCC4-S2 constructs lacked the peptide sequence encoded by this exon. When expressed in HEK-293 cells, both KCC4-S1 and KCC4-S2 mediated significant N-ethylmaleimide-stimulated, furosemide-sensitive 86Rb influx (data not shown). Western blot analysis indicated that HEK-293 cells expressing rat KCC2 and chicken KCC4-S1 contained one major band detected by rb-B22-KCC2 (Fig. 5). In contrast, this antibody detected no protein in cells expressing chicken KCC4-S2. Our results confirm that rb-B22-KCC2 recognizes an epitope encoded by exon 22 but not exon 23 of chicken KCC4 and support the hypothesis that the protein recognized by rb-B22-KCC2 in chicken heart was KCC4.

The rb-B22-KCC2 antibodies were generated against a fusion protein (rat KCC2: Ser-932 to Asn-1043) that includes only two short regions with significant identity to chicken KCC4 (Fig. 6A). The first 13 amino acids of this fusion protein are 92% identical (12 of 13 amino acids) to chicken KCC4, and the last 9 amino acids are 89% identical (8 of 9 amino acids) to chicken KCC4. To further test the hypothesis that rb-B22-KCC2 cross-reacts with KCC4 in chicken heart, two separate blocking peptides were prepared: S932ITDESRGSIRRK (13-mer; Fig. 6A) and MKPEWENLN1043 (9-mer; Fig. 6A). Membranes prepared from stable HEK-293 cells expressing rat KCC2 or chicken KCC4-S1 were then probed following preadsorption of rb-B22-KCC2 with increasing amounts of purified 13-mer peptide. Although the 13-mer peptide did not prevent KCC2 labeling, it eliminated KCC4-S1 recognition when added in amounts exceeding 1 μg (Fig. 6B). Some decrement in KCC2 signal was noted following preadsorption with the 13-mer peptide, suggesting that a subset of antibodies within the polyclonal mixture recognized the 13-mer peptide and were removed from the reactive pool. To test whether chicken heart expresses KCC4, similar blots were probed with rb-B22-KCC2 before and after preadsorption with 10 μg of the 13-mer peptide. Control blots confirmed that the rb-B22-KCC2 antibodies strongly recognizes a 140- to 145-kDa band from HEK-293 cells expressing rat KCC2 or chicken KCC4-S1 as well as chicken heart, along with a slightly smaller protein from chicken brain (Fig. 6C). The band at >240 kDa observed in some of the lanes is often seen with the KCCs and appears to represent oligomerization of these proteins (6). Preadsorption of rb-B22-KCC2 with 10 μg of the 13-mer peptide eliminated recognition of only KCC4-S1 (Fig. 6D). As noted for KCC2 in Fig. 6B, the 13-mer peptide also modestly reduced recognition of the ~140- to 145-kDa protein for KCC2, chicken brain, and chicken heart (Fig. 6D). A similar approach was used to evaluate the effect of the 9-mer blocking peptide. Preadsorption of the rb-B22-KCC2 antibodies with 10 μg of the 9-mer peptide, either alone (Fig. 6E) or in combination with the 13-mer peptide (Fig. 6F), did not interfere with its recognition of KCC2, KCC4-S1, or KCC4 proteins in chicken brain and heart. These data are inconsistent with the hypothesis that the protein recognized by rb-B22-KCC2 in chicken heart is KCC4 and support the alternative hypothesis that the protein in chicken heart is, in fact, KCC2. These data establish that KCC2 protein is expressed at significant quantities in a native tissue outside the CNS of a vertebrate.

Identification and quantification of KCC1, KCC2, and KCC4 transcripts in chicken tissues. The expression of KCC2 transcript in various chicken tissues was evaluated using RT-PCR. Gene-specific primers for chicken KCC2 were designed to target sequence in adjacent exons to avoid genomic DNA artifacts. Robust KCC2 transcript expression was identified in brain and heart with much weaker expression in colon, liver, and skeletal muscle (Fig. 7A). Additional experiments were undertaken to explore the relative expression levels of the KCC isoforms in chicken heart and brain using real-time PCR. These studies required that we first identify the sequence and clone chicken KCC1 and KCC2. This was accomplished using a combination of database searching and PCR (see MATERIALS AND METHODS). Gene-specific primer sets for chicken KCC1, KCC2, and KCC4 were designed to 1) target sequence in the region of the last 6 exons of each isoform, 2) span adjacent exons to avoid genomic DNA artifacts, and 3) avoid alternatively spliced exons (Table 1). We first tested the specificity of each primer set and confirmed the presence of a single amplification product from 10-amino acid c-myc peptide. Western blots panels were probed with either the rb-B22-KCC2 antibodies (left; 1:2,000) or the c-myc peptide monoclonal antibody (right; 1:2,000).

Fig. 5. Cross-reactivity of the rb-B22-KCC2 antibodies with chicken KCC4. Membranes were prepared from untransfected HEK-293 cells (control) and HEK-293 cells stably expressing either the rat KCC2 protein, chicken KCC4-S1, or chicken KCC4-S2 protein. Each of the expression constructs for the rat KCC2, chicken KCC4-S1, and chicken KCC4-S2 proteins were epitope tagged with the 10-amino acid c-myc peptide. Western blots panels were probed with either the rb-B22-KCC2 antibodies (left; 1:2,000) or the c-myc peptide monoclonal antibody (right; 1:2,000).
One question that still remained was the distinct size difference between the KCC2 protein observed in chicken brain and chicken heart that we consistently detected on Western blots with our KCC2 antibodies. In Fig. 4, we demonstrated that the size difference could be explained by differences in core protein mass. Uvarov et al. (41) reported that the mammalian KCC2a and KCC2b (41).

Expression of chicken KCC2a or KCC2b in HEK-293 cells resulted in a significant furosemide-sensitive 86Rb influx, and this activity was further stimulated after treatment with N-ethylmaleimide (data not shown). A high constitutive activity has likewise been reported for mammalian KCC2a and KCC2b (41).

Chicken KCC2a encodes a core protein that is larger than chicken KCC2b by ~2 kDa, which corresponds to the size difference we detected on Western blots for KCC2 in chicken heart whereas KCC2b predominates in chicken brain. While the KCC2 mRNA sequence (XM001236721) identified in the NCBI database correctly coded for exons 2–26, the first 136-bp size difference we detected on Western blots for KCC2 could be explained by differences in core protein mass. Uvarov et al. (41) reported that the mammalian KCC2a and KCC2b (41).

Expression of chicken KCC2a or KCC2b in HEK-293 cells resulted in a significant furosemide-sensitive 86Rb influx, and this activity was further stimulated after treatment with N-ethylmaleimide (data not shown). A high constitutive activity has likewise been reported for mammalian KCC2a and KCC2b (41).
difference we noted on Western blots for KCC2 in chicken heart and brain. To test the hypothesis that adult chicken heart and brain differentially expressed the two splice variants of KCC2 (KCC2a and KCC2b), real-time PCR was performed using primers specific for each of the two alternate first exons of chicken KCC2 (Table 1). As shown in Fig. 7C, chicken brain expressed predominantly KCC2b with KCC2a making up a minor fraction of the total KCC2 in chicken brain. In chicken heart, the only transcript that could be detected was that of KCC2a. These semiquantitative real-time PCR data are consistent with our protein data and support the hypothesis that the predominant form of KCC2 in chicken heart is KCC2a, while chicken brain expresses mainly the KCC2b splice variant.

Immunolocalization of KCC2 protein in adult chicken heart.

To identify the type of cells in chicken heart that express KCC2, tissues isolated from the left ventricle of adult chicken were probed with two different KCC2 antibodies prepared against the rat B22-KCC2 fusion protein, one prepared in rabbit (rb-B22-KCC2) and another in guinea pig (gp-B22-KCC2). Both rb-B22-KCC2 and gp-B22-KCC2 antibodies recognized the same bands on Western blots (data not shown). Prominent labeling was observed along the margin of cells distinguished as cardiomyocytes by colabeling with cardiac troponin-I (Fig. 9, left). In addition to this prominent plasma membrane labeling, a variable degree of punctate intracellular labeling of cardiomyocytes was noted with both KCC2 antibodies (Fig. 9). The patterns of labeling observed with the two independently generated KCC2 antibodies were similar and largely overlapping with those observed with an antibody against Na,K-ATPase-a subunit (Fig. 9, right). Similar labeling was observed in right ventricle (data not shown). To confirm the specificity of rb-B22-KCC2 and gp-B22-KCC2 to KCC2 in the chicken heart, both antibodies were preadsorbed with the 13-mer peptide shown to block recognition of KCC4-S1 (Fig. 6). The pattern of labeling with or without preadsorption was indistinguishable (data not shown). These results demonstrate that KCC2 is exceptionally abundant in the plasma membrane of cardiomyocytes throughout the chicken ventricle.
DISCUSSION

In this study, biochemical and molecular analyses were employed to characterize a K\(^{+}\)-Cl\(^{-}\) cotransporter in avian cardiomyocytes. Using antibodies previously prepared against a rat KCC2 fusion protein (46), we demonstrated strong recognition of a 140- to 145-kDa glycoprotein in membranes prepared from chicken brain and chicken heart but not at significant levels in any other chicken tissue. Preabsorption of the antibodies with 9 and 13 amino acid peptides (prepared over the only two extended regions of the entire fusion protein displaying significant identity between rat KCC2 and the other chicken KCC isoforms) demonstrated that the antibodies specifically recognize KCC2 in chicken brain and heart on Western blots. RT-PCR and real-time PCR identified and quantified transcript expression of two alternatively spliced variants of KCC2 in adult chicken brain and heart. In earlier studies, Uvarov et al. (41) demonstrated that the mammalian KCC2 gene contains two alternate first exons each under control by separate promoters, leading to two splice variants (KCC2a and KCC2b) that differ only in the amino-terminus encoded by these two alternate first exons. Our identification of both of these KCC2 splice variants in chicken demonstrates the presence of a similar KCC2 gene structure in the avian class. In adult mouse brain, KCC2b was the predominant form whereas KCC2a made up <10% of the total KCC2 transcripts (41). Similarly, KCC2a transcripts in adult chicken brain comprised <10% of the total KCC2 transcript levels (Fig. 9). Uvarov et al. (41) did not detect significant expression of either KCC2a or KCC2b transcripts outside the CNS and concluded that both KCC2 splice variants were restricted to CNS neurons. Significantly, we found that while KCC2b was most highly expressed in chicken brain, KCC2a was the most prevalent, if not exclusive, splice variant in chicken heart. Consistent with our finding of a slightly larger (~2–5 kDa) KCC2 protein in heart (where KCC2a predominates) than brain (where KCC2b predominates), chicken KCC2a encodes a core protein that is ~2 kDa larger than that encoded by chicken KCC2b. Immunofluorescent microscopy using two independently generated antibodies revealed specific expression of KCC2 protein in the plasma membrane of cardiomyocytes throughout the chicken ventricles.

Expression of KCC2 outside the CNS. While it is generally believed that KCC2 is expressed only in the CNS, the finding of KCC2 transcript outside the CNS is not unprecedented. KCC2 transcript expression has been reported in a number of different mammalian cultured cells, including rat vascular smooth muscle cells (13), human osteoclasts (9), human lens epithelial cells (25, 26), and several human cancer cell lines (45). All of the studies to date that have detected KCC2 outside the CNS have come from studies using mammalian cultured cells. Our study, however, demonstrates that robust KCC2 transcript and protein expression is present in native tissue outside the vertebrate CNS. In the one study where data were presented examining expression of both KCC2 splice variants, KCC2a was the only variant detected in human lens epithelial cells (25). Based on our data in chicken and those of Lauf et al. (25), we suspect that KCC2b is primarily expressed in the CNS, whereas KCC2a exhibits a broader vertebrate tissue distribution.

Functional characterization of K\(^{+}\)-Cl\(^{-}\) cotransport in chicken cardiomyocytes. Our finding of robust KCC2 transcript and protein in chicken cardiomyocytes is consistent with earlier evidence for KCC activity in cultured chicken cardiomyocytes (35). In this preparation, chick embryonic hearts were disag-
The cardiac cycle. Hence, regular and sustained alterations in polarity during the course of neurons, the membrane voltage of cardiomyocytes undergoes electrical activity (14). Currents through these Cl channels the ability to contribute significant inward (depolarizing force for Cl movement through the channels) and hence on [Cl\textsuperscript{−}]. In order for cardiomyocytes to regulate [Cl\textsuperscript{−}], effectively, they must employ both Cl accumulation and Cl extrusion transport pathways. Chloride accumulation transporters identified in the heart include Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport (NKCC1) and anion exchange (AE3, SLC26a6), both of which appear to be responsible for maintaining cardiomyocyte [Cl\textsuperscript{−}], above electrochemical equilibrium under quiescent conditions (2, 37, 40, 42–44). In contrast, Cl extrusion transporters in cardiomyocytes have been largely ignored despite the fact that Cl\textsuperscript{−} loading during the sustained depolarized plateau phase of the action potential is likely the greatest threat to Cl\textsuperscript{−} homeostasis in the beating cardiomyocyte. Because of the relatively long action potential of cardiomyocytes (compared with nerve and skeletal muscle), the forces favoring net Cl\textsuperscript{−} influx are sustained during the plateau phase. Chloride accumulation is of particular concern at high heart rates with β-adrenergic stimulation as the membrane voltage spends more time in the depolarized state where the outwardly rectified currents through CaCC (I\textsubscript{CaCC} activated by increased Ca\textsuperscript{2+} transient) and CFTR (I\textsubscript{CFTR} activated by increased cAMP) are enhanced (i.e., greater conductance and larger driving force). The physiological significance of enhanced I\textsubscript{CaCC} and I\textsubscript{CFTR} at high heart rates with β-adrenergic stimulation appears to be related to their ability (along with PKA activation of K\textsuperscript{+} current) to help offset the Ca\textsuperscript{2+} current-induced prolongation in action potential duration, thus permitting sympathetic stimulation to increase contractility without disturbing normal conduction patterns (17). If this Cl\textsuperscript{−} load is not counteracted appropriately by Cl\textsuperscript{−} extrusion, the resulting [Cl\textsuperscript{−}] increase will reduce the ability of I\textsubscript{CFTR} and I\textsubscript{CaCC} to help curtail action potential prolongation (18, 23). Such an elevation of [Cl\textsuperscript{−}], and the associated positive shift in the Cl\textsuperscript{−} reversal potential may result in early afterdepolarizations and contribute to arrhythmogenesis (47). To maintain Cl\textsuperscript{−} homeostasis, the channel-mediated Cl\textsuperscript{−} influx during β-adrenergic stimulation must be countered by a Cl\textsuperscript{−} extrusion mechanism that ideally is electroneutral and will therefore not disrupt normal electrical activity. Given our identification in this report of robust expression of KCC2 in chicken cardiomyocytes, we hypothesize that this cotransporter is critical for effective Cl\textsuperscript{−} homeostasis especially under conditions of elevated heart rate with β-adrenergic stimulation.

In this study, we presented evidence for the presence of KCC2 in both CNS neurons and ventricular cardiomyocytes of chicken. In mammals, however, KCC2 appears to be restricted to central neurons as it exhibits no measurable expression outside the CNS. The notable appearance of KCC2 in chicken heart is yet another example of the remarkable evolution of the KCCs that has taken place within the vertebrate classes. We have identified a number of other examples of the evolution of the KCCs among the vertebrates, including class-specific exon deletion, alternative splicing of exons, and apparent gene deletion. Until additional studies are performed examining the tissue expression patterns of KCC2 in more mammals, birds, and lower vertebrates, it is difficult to speculate whether there...
has been an evolutionary gain of KCC2 in avian cardiomyocytes or evolutionary loss of KCC2 in mammalian cardiomyocytes. It is possible that the presence of KCC2 may correlate with the physiological demands of heart tissue (e.g., heart rate and force generation). While the role of KCC2 in mammalian cardiomyocytes represents a nascent area of research.

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