Identification of Key Functional Domains in the C Terminus of the K\(^{+}\)-Cl\(^{-}\) Cotransporters*

Marc J. Bergeron, Edith Gagnon, Luc Caron, and Paul Isenring

From the Nephrology Research Group, L’Hôpital-Dieu de Québec Institution, Department of Medicine, Faculty of Medicine, Laval University, Québec G1R 2J6, Canada

The K\(^{+}\)-Cl\(^{-}\) cotransporter (KCC) isoforms constitute a functionally heterogeneous group of ion carriers. Emerging evidence suggests that the C terminus (Ct) of these proteins is important in conveying isoform-specific traits and that it may harbor interacting sites for 4β-phorbol 12-myristate 13-acetate (PMA)-induced effectors. In this study, we have generated KCC2-KCC4 chimeras to identify key functional domains in the Ct of these carriers and single point mutations to determine whether canonical protein kinase C sites underlie KCC2-specific behaviors. Functional characterization of wild-type (wt) and mutant carriers in Xenopus laevis oocytes showed for the first time that the KCCs do not exhibit similar sensitivities to changes in osmolality and that this distinguishing feature as well as differences in transport activity under both hypotonic and isotonic conditions are in part determined by the residue composition of the distal Ct. At the same time, several mutations in this domain and in the proximal Ct of the KCCs were found to generate allosteric-like effects, suggesting that the regions analyzed are important in defining conformational ensembles and that isoform-specific structural configurations could thus account for variant functional traits as well. Characterization of the other mutants in this work showed that KCC2 is not inhibited by PMA through phosphorylation of its canonical protein kinase C sites. Intriguingly, however, the substitutions N728S and S940A were seen to alter the PMA effect paradoxically, suggesting again that allosteric changes in the Ct are important determinants of transport activity and, further, that ionic strengths are also important in regulating transport activity

1 The abbreviations used are: CCC, cation-Cl\(^{-}\) cotransporter; Ct, C terminus; Nt, N terminus; FR, flux rate; KCC, K\(^{+}\)-Cl\(^{-}\) cotransporter; L medium, low Cl\(^{-}\)/isotonic medium; LH medium, low Cl\(^{-}\)/hypotonic medium; ms, mouse; NKCC, Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter; OA, okadaic acid; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; R medium, regular medium or normal Cl\(^{-}\)/isotonic medium; r/t, rat; wt, wild type; JNK, c-Jun NH\(_{2}\)-terminal kinase; Tricine, N-(2-hydroxyethyl)glycine.

2 This work was supported by the Kidney Foundation of Canada and the Canadian Institute of Health and Research (Grants MOP-68949 and MOP-15405). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 Fonds de la Recherche en Santé du Québec scholar.

4 A professor of Medicine at Laval University and holder of a Canada Research Chair in Molecular Physiology. To whom correspondence should be addressed: L’Hôpital-Dieu de Québec, du CHUQ, 10 Rue McMahon, Québec G1R2J6, Canada. Tel: 418-691-5151 (ext. 15477); Fax: 418-692-5795; E-mail: paul.isenring@crhdq.ulaval.ca.

5 The abbreviations used are: CCC, cation-Cl\(^{-}\) cotransporter; Ct, C terminus; Nt, N terminus; FR, flux rate; KCC, K\(^{+}\)-Cl\(^{-}\) cotransporter; L medium, low Cl\(^{-}\)/isotonic medium; LH medium, low Cl\(^{-}\)/hypotonic medium; ms, mouse; NKCC, Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter; OA, okadaic acid; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; R medium, regular medium or normal Cl\(^{-}\)/isotonic medium; r/t, rat; wt, wild type; JNK, c-Jun NH\(_{2}\)-terminal kinase; Tricine, N-(2-hydroxyethyl)glycine.

6 M. J. Bergeron and P. Isenring, unpublished results.

Received for publication, January 3, 2006, and in revised form, March 23, 2006 Published, JBC Papers in Press, April 4, 2006, DOI 10.1074/jbc.M600015200

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
KCC2 is conveyed through a unique 15-residue stretch (amino acids 1021–1035) in the distal Ct. For this reason, they termed the stretch in question the "ISO segment."

In this work, the role of the Ct was explored further by analyzing distinctive sets of KCC2-KCC4 chimeras as well as selected mutants in which putative PKC sites were altered. Our studies led to the identification of protein segments that play unique functional roles but not that of the underlying the PMA effect through canonical PKC phosphorylation sites. Our studies have also revealed that the Ct may impart isoform-specific functional traits by adopting defined conformational ensembles and that, for KCC2, such ensembles are probably more important than the ISO segment.

**EXPERIMENTAL PROCEDURES**

Chemicals, reagents, or kits were from several suppliers. They included: ^86^RbCl (PerkinElmer Life Sciences), the mouse anti-c-Myc monoclonal antibody (Roche Applied Science), the horseradish peroxidase-conjugated sheep anti-mouse anti-IgG and the Alexa Fluor antibody (Roche Applied Science), the horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce), as well as various salts, sucrose, ouabain, furinamide, bumetanide, PMD, 4e-PMDA, okadaic acid (OA) and oligonucleotides (Sigma). Vectors and constructs were all propagated in XL1 blue cells (Stratagene).

**KCC Constructs**—A total of sixteen different cDNAs was used. They were subcloned in the vector pGEM-HE or Pol1, which are designed to generate cRNA off of cDNA inserts and to increase the stability and translatability of transcription products in Xenopus laevis oocytes. Both these vectors contain a T7 promoter, a cloning site flanked by the X. laevis -globin-untranslated regions, a poly(A) tract, and an NheI linearizing site. In all experiments, cDNA amplification and mutagenesis were carried out using the pGEM-HE- or Pol1-based plasmids.

**c-myc-tagged Wild-type (wt) Rat (rt) KCC2 (KCC2^wt^) and wt Mouse (ms) KCC4 (KCC4^wt^)—**These constructs, provided to us by Dr. Eric Delpire and coworkers (Vanderbilt University, Nashville, TN), were generated from wt rtKCC2/pol1 and wt msKCC4/pGEM-HE. The insert of the first construct consists of a cDNA that contains 2306 bp of open reading frame and more than 373 bp of untranslated regions, whereas that of the other construct consists of a cDNA that contains 3252 bp of open reading frame and >250 bp of untranslated regions. Before adding the c-myc tag to wt rtKCC2, the insert was moved from pPol1 to XbaI-HindIII fragment.

**TABLE 1**

**Oligonucleotides**

Synthetic oligonucleotides were used for sequencing or to generate c-myc epitope tags and to introduce single point substitutions in KCC2/wt (A) or KCC4/wt (B). They are all written 5’ to 3’. Capital letters designate codons that were substituted or inserted. Note that for KCC4/wt, the oligonucleotides of "c-myc tag step 1" were used to create the tag per se, and those of "c-myc tag step 2", to generate an in-frame tag-KCC4 coding sequence. Also note that several of the oligonucleotides that were used to generate substitutions served as sequencing primers as well to verify whether substitutions at other sites were correctly introduced.

![Image](https://example.com/image.png)
c-Myc tag and possess single-stranded cohesive ends XbaI-NarI-compatible (wt KCC2/Po1) or Xmal-Drdl-compatible (KCC4/pGEM-HE). The c-myc-tagged wt msKCC4/pGEM-HE construct was modified once more by removing nucleotides between c-myc and the first ATG to generate an in-frame tag carrier-coding sequence. The latter modification was carried out through pairs of oligonucleotides (Table 1) using the QuikChange mutagenesis kit (Stratagene).

Tagged Chimeras: KCC2 4-2-2, KCC2 2-4-2, KCC2 2-2-4, KCC4 2-4-4, and KCC4 4-4-2. These mutants were engineered by fragment exchange after creating silent restriction sites in the C-terminal domains of KCC2wt and KCC4wt using the QuikChange mutation kit and pairs of oligonucleotides (Table 1). The three sites used to generate junction points were Bst1107I, AvrII, and EcoRV at bp 2046–2051, 2401–2406, and 2823, 2828–2826 in the KCC4wt open reading frame. In KCC2wt, they correspond to residues Gly644/Ile645, Leu763/Gly764, and Asp897/Asn898, respectively. The nomenclature used to identify the chimeras is explained under “Experimental Procedures.”

Expression of KCCs—Defolliculated stage V–VI oocytes were generally each injected with ~0.1 to 0.2 ng of KCC2wt, KCC4wt, or mutant KCCderived cRNAs, and maintained for ~3 days at 18°C in Barth’s (B) medium (Table 2) plus 125 μM furosemide. In each of the conditions tested, groups of oocytes were also injected with H2O alone to determine the component of fluxes that is due to endogenous KCC-mediated cotransport.

Functional Studies—As in previous studies by us and other groups (4, 21, 39, 40), KCC activity was assessed through influx assays. The isotope 86Rb+ was used as tracer, and all experiments were carried out at ~22°C with several types of media (Table 2) adjusted to a pH of 7.4. One of these mediums, called low Cl– (hypotonic), is hypoosmolar relative to the KCl/H2O plasma, whereas three of these media, called low Cl– (or L), normal Cl– (R) and wash (W), are approximately isoosmolar. In one experiment, two other groups of media, termed low Cl–/osmolality variable (O1 and O2) were used to determine the sensitivity of certain carriers to hypoosmotic swelling at 5 mM Cl– (KCC2wt, KCC4wt, KCC2 2-2-4, and KCC4 4-4-2) and at 55 mM Cl– (KCC2 4-2-2 and KCC4 4-2-4). For all of these media, succrose was used to adjust osmolality, whereas gluconate and/or SO42− were used as replacement of Cl–.

Before the flux assay, furosemide was first removed through rinses in large volumes of medium B. Next, oocytes were subjected to two consecutive incubation steps, one of variable duration in medium R, L, or O1. O2, or O3, plus 0.1–1.0 mM ouabain, with or without 250 μM furosemide, with or without 0.1–1.0 mM PMDA or 4(nitro-PMDA, with or without 0.5 μM OA) and another of 45 min in medium R (plus 0.125 μCi/mg 86Rb+ plus 0.1 μM ouabain), with or without 5 mM bumetanide, with or without 250 μM furosemide. It should be noted that, in most studies, bumetanide was added at low concentrations during the second incubation to inhibit Na+-K+-Cl– cotransport while leaving K+–Cl– cotransport intact (4, 21). Fluxes were ended with several rinses in medium W (plus 0.1 μM ouabain, plus 250 μM furosemide, plus 250 μM bumetanide), and oocytes were transferred to 96-well plates prefilled with 2% SDS and scintillation fluid. After a
brief stabilization period, $^{86}$Rb$^+$ was detected with the TopCountNXT counter (Packard Instrument Co.).

For each condition tested, counts among 3–12 oocytes (typically ~8 oocytes) were averaged and transposed into flux rates (FRs) based on the equation: $\text{FRs} = (\text{counts/oocyte} \times [^{86}\text{Rb}^+])$ in flux medium $\div ([\text{counts}] \times \text{incubation time in flux medium})$, assuming that membrane surface areas among stage V–VI oocytes are the same. In all experiments, FRs measured in KCC-expressing oocytes were also converted into cotransporter-specific FRs (FRs$\text{KCC}$), where FRs$\text{KCC} =$ (FRs without furosemide for KCC-expressing oocytes $-$ FRs with furosemide for KCC-expressing oocytes) $-$ (FRs without furosemide for H$_2$O-injected oocytes $-$ FRs with furosemide for H$_2$O-injected oocytes), or they were converted into background-subtracted FRs, where FRs$\text{KCC} =$ (FRs without furosemide for KCC-expressing oocytes $-$ FRs with furosemide for H$_2$O-injected oocytes). Note that when calculated FRs were from experiments in which the effect of phorbol esters or a change in osmolality was assessed, they were converted a third time by normalizing them to the equation: $\text{FRsKCC} = \text{FRs} \div \text{osmolality}$. When appropriate, differences between groups of variables were analyzed by Student two-tail $t$-tests, rejecting the null hypothesis for $p > 0.05$.

RESULTS

Preamble—Cell swelling was induced in the current study with a 5 mM Cl$^-$, 125 mosM solution (medium LH), and its effect on KCC activation was determined by comparing flux rates (FRs) measured after this maneuver to those measured after a 5 mM Cl$^-$, 200 mosM solution (medium L). The reason for reducing external Cl$^-$ (Cl$^-_o$) to low levels was to minimize differences in Cl$^-_o$ between cells incubated in the hypotonic versus control solution and, accordingly, Cl$^-_o$-dependent differences in KCC activity, as could occur based on the positive correlation that exists between the latter two variables (21–23). Given, however, that medium LH could still lead to slightly lower Cl$^-_o$ than medium L by diluting cytosolic ions through cell swelling (21, 45, 46), additional studies were performed to verify whether changes in ion transport, if they arose, resulted from changes in cell volume or Cl$^-_o$, assuming that medium L acts by decreasing anion concentration. We suspect that this is the case based on previous work (47) and on the levels of Cl$^-_o$ to which $^{86}$Rb$^+$-containing flux media were set in the current work. These additional experiments consisted of FR measurements obtained after these procedures, cells were transferred to a lysis solution called medium X (20 mM Tris, pH 8.0, 1 mM EDTA, 4 mM MgCl$_2$, 80 mM sucrose, 10% glycerol, 1% Triton-X, 1 mM phenylmethylsulfonyl fluoride) and homogenized mechanically through pipette tips. Oocyte extracts were then incubated in this medium for another 30 min at 4 °C after which they were cleared by centrifugation and brought to 1 ml in medium X. Cell-surface biotinylated proteins were extracted from this medium after adding 50 µl of agaro-coupled streptavidin and pelleting beads by centrifugation for 1 h, and they were released from the pellets in heated protein sample buffer. For the Western analyses, biotinylated proteins were migrated on 7.5% SDS-polyacrylamide Tricine gels, transferred onto Immobilon-P membrane blots (Millipore), and revealed by chemiluminescence using the Amersham Biosciences ECL solutions after sequential incubations of the blots with a primary and secondary antibody.

Software and Statistical Analyses—DNA characterizations were performed by automated sequencing, using plasmid- or KCC-derived primers (Table 1), and by restriction analyses. For BLAST searches, sequence alignments, and structure predictions, we used a combination of programs, including PLOT (B. Forbush) and DNAStar (Lasergene). When appropriate, differences between groups of variables were analyzed by Student two-tail $t$-tests, rejecting the null hypothesis for $p > 0.05$.

Role of the C Terminus in Ion Movement by the KCCs

| TABLE 2 | Composition of flux solutions |
|----------------|-----------------------------|
| **Medium** | **Solution** | **[ion]** | **Osmolarity** |
| R | Normal Cl$^-$/isotonic | Na$^+$ | 87 | 5 | 86 | 2 | 2 | 1 | 1 | 10 | 0 | 0 | 194 |
| L | Low Cl$^-$/isotonic | Rb$^+$ | 56 | 3.2 | 5.2 | 1.3 | 1.3 | 0.6 | 0.6 | 6.4 | 50 | 69 | 194 |
| LH | Low Cl$^-$/hypotonic | Cl$^-$ | 56 | 3.2 | 5.2 | 1.3 | 1.3 | 0.6 | 0.6 | 6.4 | 50 | 0 | 125 |
| LOa | Low Cl$^-$/variable osm | Ca$^{2+}$ | 56 | 3.2 | 5.2 | 1.3 | 1.3 | 0.6 | 0.6 | 6.4 | 50 | 0 | 125 |
| Oa | Normal Cl$^-$/variable osm | Mg$^{2+}$ | 56 | 3.2 | 5.2 | 1.3 | 1.3 | 0.6 | 0.6 | 6.4 | 50 | 0 | 125 |
| W | Wash medium | PO$_4^{3-}$ | 19 | 73 | 8 | 2 | 2 | 1 | 1 | 10 | 78 | 0 | 194a |
| B | Barth medium | SO$_4^{2-}$ | 100 | 1 | 90 | 0.7 | 0.8 | 0 | 0.8 | 10 | 78 | 0 | 207a |

[a] HEP, HEPES; GLUC, gluconate; and SUC, sucrose.
[b] K$^+$ was used instead of Rb$^+$.
incubations in a 86 mM Cl\(^-\), 200 mosM solution (medium R) and compared with measurements obtained after incubations in medium L.

**Synthesis of wt and Mutant KCCs in X. laevis Oocytes**—Carrier expression levels and distributions were analyzed through immunofluorescence studies using oocytes that had been maintained in isotonic media before membrane permeabilization and through Western analyses of cell-surface-biotinylated KCCs using oocytes that were subjected to different preincubations (in medium LH, L, or R). Immunofluorescence data are depicted in Fig. 2A using representative fields among ≥2 oocytes for each of the KCCs expressed (panels 1–16) and Western analyses in Fig. 2B through duplicate experiments using four different KCCs (panels 1 and 2).

In the upper group of images (Fig. 2A), it is seen that the c-Myc antibody labels all of the heterologous proteins tested strongly (panels 1–16) and that it does so specifically, as revealed by the absence of signal in the H\(_2\)O controls (panels 17 and 18). It appears, moreover, that the signal is localized primarily at the cell surface and is of similar intensity among the KCC-expressing oocytes. In the lower group of images (Fig. 2B), it is seen there that differences in expression at the cell surface among the KCCs tested (KCC2\(_{w+t}\), KCC4\(_{w+t}\), KCC2\(_{2-2-4}\), and KCC4\(_{2-2-4}\)) are indeed not important. For KCC2\(_{w+t}\) and KCC4\(_{2-2-4}\), the bands are in fact of slightly fainter density but as will be shown below, transport rates by these carriers are not lower than those by KCC4\(_{w+t}\) and KCC2\(_{2-2-4}\). The Western analyses presented in Fig. 2B also reveal convincingly that KCC expression at the cell surface does not vary considerably among the preincubation conditions used.

**Sensitivity of KCC2\(_{w+t}\) and KCC4\(_{w+t}\) to Changes in Osmolality and Cl\(^-\)**—In these studies, transport was assessed following 60-min incubations in media L\(_{O2}\) (5 mM Cl\(^-\), 125–250 mosm) or media O\(_3\) (55 mM Cl\(^-\), 125–250 mosm). Results, which are expressed as background-subtracted 86Rb\(^+\) FRs normalized to those measured with 125 mosM media, are shown in Fig. 3 using panel A for condition “LO\(_2\)”, and B for “O\(_3\)”. As can be seen in both these panels, interestingly, KCC2\(_{w+t}\) is more sensitive to a decrease in osmolality than is KCC4\(_{w+t}\), at 150 mosM, e.g. KCC2\(_{w+t}\) is nearly fully stimulated while KCC4\(_{w+t}\) is less than half fully stimulated. Compared with KCC4\(_{w+t}\), conversely, KCC2\(_{w+t}\) is constitutively more active at higher osmolalities when Cl\(^-\) is 55 mM (panel B) instead of 5

---

**FIGURE 2.** Immunofluorescence studies and Western analyses of X. laevis oocytes expressing wt and mutant KCCs. A, the carriers were immunolocalized with the mouse anti-c-Myc antibody, and signals were microphotographed under epifluorescence microscopy using comparable exposure times and representative membrane sections among ≥2 oocytes. 1 and 5, KCC2\(_{w+t}\) and KCC4\(_{w+t}\)-injected oocytes; 2–4, KCC2\(_{4-2}\), KCC2\(_{w+t}\) and KCC2\(_{2-4}\)-injected oocytes; 6–8, KCC4\(_{4-2}\), KCC4\(_{w+t}\), and KCC4\(_{2-4}\)-injected oocytes; and 9–16, KCC2\(_{2-4}\), KCC2\(_{w+t}\), KCC2\(_{2-4}\), KCC4\(_{2-4}\), and KCC2\(_{w+t}\) injected oocytes. In 17 and 18, oocytes were injected with H\(_2\)O alone. In each micrograph, the cytoplasm is situated on the right-hand side. B, carriers (KCC2\(_{w+t}\), KCC4\(_{w+t}\), KCC2\(_{2-2-4}\), and KCC4\(_{2-2-4}\)) were prepared by cell-surface biotinylation and revealed through Western analyses using the same mouse anti-c-Myc antibody. Two representative gels are shown in panels 1 and 2.

**FIGURE 3.** Sensitivity of KCC2\(_{w+t}\) and KCC4\(_{w+t}\) to changes in external osmolality. After 60-min incubations in media L\(_{O2}\) (5 mM, osmolality 125, 150, 175, 200, or 250 mosM; panel A) or after 60-min incubations in media O\(_3\) (55 mM, osmolality 125, 150, 175, 200, or 250 mosM; panel B), oocytes expressing KCC2\(_{w+t}\) or KCC4\(_{w+t}\) were assayed for 86Rb\(^+\) influx measurements (alongside with H\(_2\)O-injected controls) in medium R plus 1–2 μCi/ml 86Rb\(^+\), plus 10 μM ouabain. Data correspond to background-subtracted 86Rb\(^+\) FRs that were normalized to the values obtained with the incubation in 125 mosM media. They are shown as averages (+S.E.) among two to four experiments (six to twelve oocytes/experiments), using the asterisk to indicate that they are significantly different statistically (p < 0.01) between the two carriers. Composition of media used for these studies is shown in Table 2.
Role of the C Terminus in Ion Movement by the KCCs

FIGURE 4. Functional characterizations of wt and chimeric KCCs. Oocytes expressing KCC2wt, KCC22-2-2, KCC22-4-4, or KCC22-4-2 (KCC2 group; left column) or KCC4wt, KCC44-2-4, KCC44-4-2, or KCC44-2-4 (KCC4 group; right column) were preincubated in medium LH (5 mM Cl, 125 mosM; panels A and B), medium L (5 mM Cl, 194 mosM; panels C and D), or medium R (86 mM Cl, 194 mosM; panels E and F), and subsequently assayed for $^{86}$Rb influx measurements (alongside with H$_2$O-injected controls) in medium R with or without 250 $\mu$M bumetanide, plus 1–2 $\mu$Ci/ml $^{86}$Rb$^+$, plus 10 $\mu$M ouabain, plus 5 $\mu$M bumetanide. Data correspond to background-subtracted furosemide-sensitive, bumetanide-insensitive $^{86}$Rb$^+$ FRs and are shown as averages ± S.E. among three to four experiments (four to twelve oocytes/experiments). *, the data are significantly different statistically compared with the wt carrier in a given panel ($p < 0.05$ for KCC44-4-4 in panel B, and $< 0.01$ in all other cases); $\dagger$, the data are significantly different statistically from all of the other values in a given panel ($p < 0.01$); and $\ddagger$, the data are not significantly different statistically from “0.” The composition of media used is shown in Table 2.

mm (panel A). These results suggest that Cl$^-$ is an important determinant of constitutive activity for KCC2wt under isotonic conditions.

Functional Characterizations of KCC2-KCC4 Chimeras—These mutants were used to determine whether certain regions within the Ct, such as those that were described just above and those identified in previous studies, confer isoform-specific functional behaviors (18, 31, 40). Preincubations were carried out in three types of solutions, media LH (5 mM Cl, 125 mosM), L (5 mM Cl, 200 mosM), or R (86 mM Cl, 200 mosM), and results are expressed (in Fig. 4) as background-subtracted furosemide-sensitive, bumetanide-insensitive $^{86}$Rb$^+$ FRs.

With medium LH (Fig. 4, panels A and B), both the wt KCCs (KCC22-2-2 and KCC44-4-4 in Fig. 4) are found to exhibit above background transport activity with FRs that are significantly different statistically from “0” but much higher for KCC2wt. As for the reciprocal chimeras, differences in FRs are also observed, but the direction and amplitude of changes among each group are quite variable compared with the wt carriers. In panel A, e.g., the activity of a KCC2 that has the proximal third of its Ct replaced by that of KCC4wt is much higher compared with KCC2wt, whereas the activity of a KCC2 that has the middle third or distal third of its Ct replaced (KCC22-4-2 and KCC22-2-4) is much lower. In panel B, FRs among the carriers tend to be less variable except for KCC44-4-4, which is much more active that the others and more active than KCC22-4-4 as well. For unknown reasons, the behavior of KCC22-4-4 in this group is not reciprocal to that of KCC22-2-2.

With medium L (Fig. 4, panels C and D), the pattern of changes in transport activity among the carriers is almost completely identical to that observed with medium LH, suggesting that the mutations have not acted solely by altering transport activity to changes in osmolality. It can be noted here that FRs are much lower than those measured with medium LH; for three of these carriers, in fact, transport activity is not different from “0” statistically. These results suggest that some of the mutations may have generated inactive carriers or that differences in FRs between cRNA-injected and H$_2$O-injected oocytes were too small to be detected in this series of assays. We favor the second hypothesis, because KCC4wt has been shown on several occasions by our group to exhibit definite transport activity (generally between 0.1 to 0.2 nmol/oocyte/h) under the “L” condition, and because all of the chimeras tested in this study were found to be active under at least one condition.

In contrast to medium L, medium R (Fig. 4, E and F) is found to induce above background furosemide-sensitive, bumetanide-insensitive FRs for all of the carriers tested except KCC44-2-4. Here again, and even if FRs measured under this condition tend to be intermediate between those measured with medium LH and those measured with medium L, the pattern of changes among the KCCs is very similar to that observed with medium LH. An obvious exception in these regards is KCC22-4-2, which is seen to display a KCC2wt-like phenotype in panel E, when it was clearly less active than the native carrier in panels A or C.

The behavior of the chimeras suggests thus far that the protein segments analyzed through various substitutions accomplish very different roles. It is noticeable that KCC22-4-2 and KCC44-2-4 are not only functionally reciprocal under all of the conditions tested but that they also adopt the functional properties of the wt carrier from which the distal Ct
Role of the C Terminus in Ion Movement by the KCCs

FIGURE 5. Detailed characterizations of four different chimeras. A and B, sensitivity of KCC2-4Δ2 and KCC2Δ2-4 to changes in external osmolality. After 60-min incubations in media LO (Cl− 5 mM, osmolalities 125, 150, 175, 200, or 250 mosM), KCC2-4Δ2 or KCC2Δ2-4-injected oocytes were assayed for 86Rb+ influx measurements (alongside with H2O-injected and KCC4wt, as well as KCC2wt injected controls) in medium R plus 1–2 μCi/ml 86Rb+, plus 10 μM ouabain. Data correspond to background-subtracted 86Rb+ FRs that were normalized to the values obtained with the incubation in 125 mosm media. They are shown as averages (± S.E.) among two to five experiments (eight to twelve oocytes/experiments), using the asterisk to indicate that these values are significantly different statistically (p < 0.01) between the two carriers. C, characterizations of KCC2-4Δ2-86Rb+ and KCC2Δ2-4 with external osmolality. These agents were shown by us and others to indicate that they are significantly different statistically (p < 0.01) between the two carriers. D, effect of PMA and OA on carrier activity. After 15-min incubations in one of two media (LH or L) with or without 250 μM furosemide, or with or without 0.1 μM OA (bars 5–8), oocytes expressing KCC2wt or mutant KCC2Δ2-4 were assayed for 86Rb+ influx measurements (alongside with H2O-injected controls) in medium R with or without 250 μM furosemide, plus 1–2 μCi/ml 86Rb+, plus 10 μM ouabain, plus 5 μM bumetanide. Data correspond to background-subtracted furosemide-sensitive, bumetanide-insensitive 86Rb+ FRs and are shown as averages (± S.E.) among three to four experiments (six to twelve oocytes/experiments). In this panel, the asterisk indicates that the data are significantly different statistically (p < 0.01) compared with the three other groups tested within the condition. In the other panel, the asterisk indicates that the data are significantly different statistically (p < 0.01) from KCC2wt. The composition of the media is as in Table 2.

originate. The chimera KCC2-4Δ2, on the other hand, exhibits a hybrid behavior between the wt carriers, and the chimera KCC2Δ2-4, a rogue behavior with FRs that are outside the range of those measured for the wt carriers. The implication of these various findings will be discussed below.

Additional Studies for KCC2-4Δ2 and KCC2Δ2-4—Such studies were carried out to determine the phenotype of certain chimeras in greater detail and whether the loss of a putative PKC site in the proximal Ct of KCC2-4Δ2, such a site is present in KCC2wt, but not in KCC4wt, accounts for heightened K+-Cl− cotransport by this chimera. In one of the mutants, an Asn → Ser substitution was created in the proximal Ct to reconstitute the PKC site, and in the other, an Asn → Asp substitution to mimic phosphorylation. Results are summarized in Fig. 5.

In Fig. 5A, the chimera KCC2-4Δ2 is seen to behave exactly as KCC4wt even if it is almost entirely composed of the KCC2wt sequence, suggesting that the distal Ct conveys increased sensitivity to changes in osmolality. In panel B, on the other hand, KCC2Δ2-4 is seen to display even more peculiar characteristics in that its sensitivity to changes in external osmolality is much higher than that of KCC2wt. Among various possibilities, these results suggest that mutations in the proximal Ct have induced allosteric effects or that they have led to the loss or gain of key regulatory sites.

The latter hypotheses were tested more specifically by analyzing the functional features of the two additional mutants KCC2Δ2-4ΔN728S and KCC2Δ2-4ΔN728D (Fig. 5G). They were also tested by analyzing the effect of PMA and OA on KCC2wt and KCC24-2-2 (shown in panel D as PMA- or OA-induced % changes in furosemide-sensitive, bumetanide-insensitive 86Rb+ FRs), given that these agents were shown by us and others (see below and Refs. 14–17 and 27–36) to decrease ion transport by certain isoforms. Note that in certain studies oocytes were preincubated
for only 15 min in medium LH, L, or R to avoid PMA- or OA-induced toxicity; as will be explained in Fig. 6, fortunately, KCC activity after this incubation time is still higher with medium LH compared with medium L.

Looking at Fig. 5C, one can observe that the Asn → Ser substitution in KCC2_{4-2-2} results in decreased K\(^+/\)Cl\(^–\) cotransport under all conditions used, and that the Asn → Asp substitution almost completely abolishes this process. Although such results could indicate that Ser\(^{728}\) in KCC2\(_{wt}\) is in fact a phosphorylation site, one can also observe by looking at panel D that PMA decreases KCC2\(_{4-2-2}\)-mediated activity much more than KCC2\(_{wt}\)-mediated activity (25–30% inhibition versus 65–70%) and that the effect of OA is quantitatively similar between KCC2\(_{4-2-2}\) and KCC2\(_{wt}\). Taken together, hence, the results of these studies suggest that the loss of a phospho-acceptor site (Ser\(^{728}\)) in the proximal C-terminal domain of KCC2 does not account for the behavior of KCC2\(_{4-2-2}\) and are consistent with the idea of mutation-induced allosteric effects. As will be shown below in Fig. 8, the consequence of certain single point substitutions in KCC2\(_{wt}\) is consistent with this conclusion as well.

**Initial Characterizations of \(wt\) and **Mutant **KCC2s in Which Putative PKC Sites Were Altered**—Theses characterizations were conducted through immunofluorescence studies, which have already been presented, and functional studies, which are presented in Fig. 6, using once more background-subtracted furosemide-sensitive, bumetanide-insensitive \(86^{Rb}\) \(^+\) FRs from several replicate experiments. Here, oocytes were all preincubated for 15 min in various media, the purpose of these studies being to study the effect of PMA on carrier function. These initial characterizations (Fig. 2A, panels I and 9–16, and Fig. 6) show that expression levels and transport activity under each of the conditions tested are similar among the various carriers with 2-fold increases in FRs between medium LH and medium L.

**Sensitivity of KCC2\(_{wt}\) to PMA under Low Cl\(^–\)/Isotonic versus Low Cl\(^–\)/Hypotonic Conditions**—As mentioned and presented earlier, studies by our group have shown that PMA can alter the activity of certain KCCs in *X. laevis* oocytes. Additional experiments were carried out here to determine whether the effect of PMA differs between the isotonic and hypotonic conditions by preincubating oocytes for 15 min in medium L (Fig. 7A) or LH (Fig. 7B) with increasing amounts of PMA or 4α-PMA (from 0.0 to 1.0 \(\mu M\)). Results are shown in Fig. 7 expressed as background-subtracted furosemide-sensitive, bumetanide-insensitive \(86^{Rb}\) \(^+\) FRs normalized to those measured with 0 \(\mu M\) PMA or 4α-PMA. Here, apparent \(K(P(MA))\) values based on a three-parameter Hill equation were 0.11 ± 0.02 \(\mu M\) for the hypertonic condition and 0.32 ± 0.04 \(\mu M\) for the isotonic condition. These values were also significantly different statistically (p < 0.01) from one another. The composition of the media is as in Table 2.
Role of the C Terminus in Ion Movement by the KCCs

When the preincubation is carried out in medium LH (Fig. 8A), both the wt and mutant KCCs are seen to behave analogously that is, their transport activity decreases by >30% with PMA. When, on the other hand, preincubation is carried out in medium L (Fig. 8B), two of the mutants are found to become paradoxically more sensitive to the inhibitory effect of PMA, that is, their transport activity decreases by >50%. These results suggest that the PMA effect involved in the carrier inhibition does not produce its effect via canonical PKC sites. They also suggest that residue 940 (or the region in which it occurs) could play an important role under isotonic conditions by defining the accessibility of other sites to PMA-dependent effectors.

DISCUSSION

In the current study, we have used a mutagenic approach to identify important functional domains and residues in the putative Ct of the Na+-independent group of CCCs. More specifically, we have determined the role of three protein segments in this domain by analyzing a series of KCC2-KCC4 chimeras in which short residue stretches were interchanged between the carriers, and we have also determined whether canonical PKC sites in KCC2Ct modulate or underlie the functional purpose of the protein segments identified. As a result of such studies, we have found that the three segments in question each play a definite role in carrier function but that transport activity by KCC2wt is not influenced through PKC phosphorylation of canonical PKC sites in the cytosolic domains of this carrier.

One of the functional domains analyzed through this study corresponds to the proximal Ct of the KCCs (residues 645–763 in KCC2wt and 665–783 in KCC4wt). Surprisingly, it was shown to harbor residues that are probably crucial in defining conformational ensembles rather than conveying isoform-specific traits. This assertion is supported by the peculiar behaviors of KCC24-2-2 and KCC24-2-2(N728S) that are consistent with the occurrence of allosteric effects due to substitutions in the proximal Ct of KCC2wt. Indeed, sensitivity to changes in osmolality and transport activity under all conditions tested were higher for KCC24-2-2 compared with KCC2wt, and although transport activity for KCC24-2-2 was decreased following the N728S substitution, the PMA effect was paradoxically more pronounced for KCC24-2-2, and it was not prevented by producing the same S728N substitution in KCC2mut. These results also imply that the KCC2 → KCC4 mutations did not alter the phenotype of KCC2mut, because a PKC site was lost in the proximal Ct.

Some of the functional traits exhibited by KCC24-2-2 raise the possibility that this chimera was expressed at higher levels and not that it was simply more active at the cell surface. Based on our immunofluorescence data, on the other hand, and because KCC24-2-2 exhibited increased sensitivity to changes in osmolality, we view this possibility as unlikely. In this study and in a recent study by Mercado et al. (40), moreover, Western analyses of various KCC2-KCC4 chimeras purified by cell-surface biotinylation showed that changes in activity for these types of mutants were apparently not associated with changes in cell-surface protein expression.

As a complement to the studies that were conducted for this analysis, we have not attempted to narrow the list of residues that may play a role in defining conformational ensembles throughout certain portions of the carrier. It is unlikely, however, that such residues would be found exclusively in the proximal Ct or, if this were the case, that they would interact with residues that only belong to this domain. These assumptions are based on the following data: 1) Mercado et al. (40) have observed that a chimera made of the Nt and central core of KCC4 and of the Ct of KCC2 also displayed increased transport activity compared with KCC2wt. Because the junction point of this chimera was similar to the one that was used for KCC24-2-2, residues proximal to amino acid 644 could thus play a structural role as well (2). We have recently found that the proximal and distal Ct of the NKCCs, which are closely related to the KCC isoforms, are capable of self-interactions (43, 49), suggesting that residues between these distant protein segments can associate with one another in the context of intramolecular and intermolecular interactions.

The second domain that was examined in the current study corresponds to the middle Ct. In this protein segment of KCC2mut, interestingly, the substitutions were found to induce subtler effects, altering transport activity only under the "LH" and "L" conditions. In fact, KCC24-2-2 behaved exactly as KCC4wt when Cl− was 5 mM and as KCC2mut when it was 86 mM, suggesting that the middle Ct encloses residues that confer differences in transport activity at lower ranges of Cl−. It should be mentioned, however, that the functional characteristics of KCC44-2-4 did not mirror those of KCC24-2-2 under the "L" condition, indicating that differences in carrier activity at lower ranges of Cl− are probably specified through additional domains. Although the substitutions that were generated in the middle Ct could have also acted by disrupting conformation, we are reassured with the idea that changes in functional traits reflect changes in behavior-defining residues by finding that the substitutions led to hybrid properties as well as FRs that were all within the range of those observed for KCC2mut or KCC4mut.

The third domain that was analyzed through this study corresponds to the distal Ct. Surprisingly, the effects of interchanging the latter protein segment between KCC2mut and KCC4mut were very different from those of interchanging the proximal or middle Ct. Indeed, both KCC24-2-4 and KCC44-4-3 assumed the phenotype of the carrier from which the distal Ct originated. For each of these mutants, in fact, the adopted transport

FIGURE 8. Effect of PMA on wt and mutant KCC2s under isotonic versus hypotonic conditions. A, after 15-min incubations in medium LH with or without 250 μM furosemide, with or without 0.5 μM PMA (panel A) or after 15-min incubations in medium LH with or without 250 μM furosemide, with or without 0.5 μM PMA (panel B), oocytes expressing wt KCC2 or mutant KCC24-4444, KCC44-2-2, KCC24-4444, KCC24-4444, KCC24-4444, or KCC44-2-2 were assayed for 86Rb uptake in medium LH with or without 250 μM furosemide, plus 1–2 μCi/ml 32P. T Test (two-tailed) was used to indicate that they were significantly different (p < 0.01) compared with the wt carrier. The composition of the media is as in Table 2.
activities (at 5 mM Cl⁻ or 86 mM Cl⁻) as well as the adopted sensitivity profiles to changes in osmolality were almost identical to those of the wt carrier. These results are consistent with our previous suggestion that differences in ion transport rates at lower ranges of Cl⁻ cannot be solely accounted for through variant compositions of the middle Ct. They also point toward the possibility that the distal Ct encloses key residues that account for through variant compositions of the middle Ct. It is reasonable to assume that those created in the distal Ct did not act simply by disrupting conformation. For example, KCC2wt-2-4 and KCC44-4-4 displayed transport rates that were once more within the ranges of KCC2wt and KCC4wt, and, in addition, their phenotypes mirrored one another near perfectly. At the same time, it may be of concern that a number of functional parameters were altered as a result of these mutations, indicating that the distal Ct could also be prone to conformational transitions when its composition is altered. In fact, substitution-induced allosteric effects could explain why KCC22-(S940A) exhibited a paradoxical increase in PMA sensitivity and why several chimeras in the Mercado et al. (40) study, mostly KCC4 structures in which short regions along the distal Ct were replaced by KCC2 wt counterparts, deployed "off range" transport activities. In this regard, however, it should be mentioned that, in contrast to KCC4wt, the distal end of these functionally peculiar structures all enclosed a number of KCC4wt residues.

In the same work by Mercado et al. (40), it was concluded that KCC2wt displays much higher transport activity than KCC4wt under isotonic conditions because of a unique 15-residue stretch (amino acids 1021–1035) in its distal Ct. For this reason, the stretch in question was called the "ISO segment" and ascribed the role of conveying constitutive activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform.

Role of the C Terminus in Ion Movement by the KCCs

KCC4wt, it is reasonable to assume that those created in the distal Ct did not act simply by disrupting conformation. For example, KCC2wt-2-4 and KCC4wt-4-4 displayed transport rates that were once more within the ranges of KCC2wt and KCC4wt, and, in addition, their phenotypes mirrored one another near perfectly. At the same time, it may be of concern that a number of functional parameters were altered as a result of these mutations, indicating that the distal Ct could also be prone to conformational transitions when its composition is altered. In fact, substitution-induced allosteric effects could explain why KCC22-(S940A) exhibited a paradoxical increase in PMA sensitivity and why several chimeras in the Mercado et al. (40) study, mostly KCC4 structures in which short regions along the distal Ct were replaced by KCC2 wt counterparts, deployed "off range" transport activities. In this regard, however, it should be mentioned that, in contrast to KCC4wt, the distal end of these functionally peculiar structures all enclosed a number of KCC4wt residues.

In the same work by Mercado et al. (40), it was concluded that KCC2wt displays much higher transport activity than KCC4wt under isotonic conditions because of a unique 15-residue stretch (amino acids 1021–1035) in its distal Ct. For this reason, the stretch in question was called the "ISO segment" and ascribed the role of conveying constitutive activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform. Based on our own data, on the other hand, we do not concur with such conclusions. Indeed, the absence of activity to the neuronal isoform.

In summary, we have shown that the distal Ct of the KCCs plays an important role in defining functional traits for this group of carriers. We have also shown that the basal structural arrangement that is assumed by a large portion of the Ct is probably important in specifying isoform-specific behaviors. One such behavior, higher levels of activity at lower levels of Cl⁻, may correspond to a unique and indispensable characteristic of the neuronal-specific isoform.

Acknowledgment—We are grateful to Micheline Noël, B.Sc. for superb technical assistance.

REFERENCES

1. Xu, J. C., Lytle, C., Zhu, T. T., Payne, J. A., Benz, E., and Forbush, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2201–2205
2. Payne, J. A., and Forbush, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4544–4548
3. Gambá, G., Saltzberg, S. N., Lombardi, M., Miyanoaishi, A., Lytton, J., Hediger, M. A., Brenner, B. M., and Hebert, S. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2749–2753
4. Gillen, C. M., Beill, S., Payne, J. A., and Forbush, B. (1996) J. Biol. Chem. 271, 16237–16244
5. Payne, J. A., Stevenson, T. J., and Donaldson, L. F. (1996) J. Biol. Chem. 271, 16245–16253
6. Mount, D. B., Mercado, A., Song, L., Xu, J., George, A. L., Delpire, E., and Gambá, G. (1999) J. Biol. Chem. 274, 16355–16360
7. Häki, K., D’Andrea, R. J., Furré, J., Crawford, J., Woollatt, E., Sutherland, G. R., Vadás, M. A., and Gambá, G. (1998) J. Biol. Chem. 273, 10661–10667
8. Race, J. E., Makhlouf, F. N., Logue, P. J., Wilson, F. H., Dunham, P. B., and Holtzman, E. J. (1999) Am. J. Physiol. 277, C1210–C1219
9. Williams, J. R., Sharp, J. W., Kumari, V. G., Wilson, M., and Payne, J. A. (1999) J. Biol. Chem. 274, 12656–12664
10. Hebert, S. C., Mount, D. B., and Gambá, G. (2004) Pflugers Arch. 447, 580–593
11. Karadakó, M. F., Byun, N., Mount, D. B., and Delpire, E. (2004) Neuroscience 123, 381–391
12. Velázquez, H., and Silva, T. (2003) Am. J. Physiol. 285, F49–F58
13. Jennings, M. L., and Adame, M. F. (2001) Am. J. Physiol. 281, C825–C832
14. Gambá, G. (2005) Physiol. Rev. 85, 423–493
Role of the C Terminus in Ion Movement by the KCCs

15. Eveloff, J., and Warnock, D. G. (1987) Am. J. Physiol. 252, F883–F889
16. Lauf, P. K. (1985) J. Membr. Biol. 88, 1–13
17. Lauf, P. K., Bauer, J., Adragna, N. C., Fujise, H., Zade-Open, A. M., Ryu, K. H., and Delpere, E. (1992) Am. J. Physiol. 263, C917–C932
18. Bergeron, M. J., Gagnon, E., Wallendorff, B., Lapointe, J. Y., and Isenring, P. (2003) Am. J. Physiol. 285, F68–F78
19. Payne, J. A. (1997) Am. J. Physiol. 273, C1516–C1525
20. Di Fulvio, M., Lauf, P. K., and Adragna, N. C. (2001) J. Biol. Chem. 276, C328–C336
21. Lytle, C., and McManus, T. (2002) Am. J. Physiol. 283, C1422–C1431
22. Gillen, C. M., and Forbush, B. (1999) Am. J. Physiol. 277, C772–C778
23. Bize, I., Guvenc, B., Robb, A., Buchbinder, G., and Brugnara, C. (1999) Am. J. Physiol. 277, C926–C936
24. Di Fulvio, M., Lincon, T. M., Lauf, P. K., and Adragna, N. C. (2001) J. Biol. Chem. 276, 21046–21052
25. Ferrell, C. M., Lauf, P. K., Wilson, B. A., and Adragna, N. C. (2000) J. Membr. Biol. 177, 81–93
26. Kim, H. D., Sergeant, S., Forte, L. R., Sohn, D. H., and Im, J. H. (1989) Am. J. Physiol. 256, C772–C778
27. Piechotta, K., Lu, J., and Delpere, E. (2002) J. Biol. Chem. 277, 50812–50819
28. Gagnon, K. B., England, R., and Delpere, E. (2006) Am. J. Physiol. 290, C134–C142
29. Strange, K., Singer, T. D., Morrison, R., and Delpere, E. (2000) Am. J. Physiol. 279, C860–C867
30. Mercado, A., Broumand, V., Zandi-Nejad, K., Enck, A. H., and Mount, D. B. (2006) J. Biol. Chem. 281, 1016–1026
31. Piechotta, K., Garbarini, N., Wallendorff, B., Lapointe, J. Y., and Isenring, P. (2003) Am. J. Physiol. 284, C365–C370
32. Gagnon, E., Bergeron, M. J., Brunet, G. M., Daigle, N. D., Simard, C. F., and Isenring, P. (2004) J. Biol. Chem. 279, 5648–5654
33. Novet, M. H., Bergeron, M. J., and Isenring, P. (2005) J. Membr. Biol. 220, 109–137