In vivo role of CLC chloride channels in the kidney

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Am J Physiol Renal Physiol 279: F802–F808, 2000.—Chloride channels in the kidney are involved in important physiological functions such as cell volume regulation, acidification of intracellular vesicles, and transepithelial chloride transport. Among eight mammalian CLC chloride channels expressed in the kidney, three (CLC-K1, CLC-K2, and CLC-5) were identified to be related to kidney diseases in humans or mice. CLC-K1 mediates a transepithelial chloride transport in the thin ascending limb of Henle’s loop and is essential for urinary concentrating mechanisms. CLC-K2 is a basolateral chloride channel in distal nephron segments and is necessary for chloride reabsorption. CLC-5 is a chloride channel in intracellular vesicles of proximal tubules and is involved in endocytosis. This review will cover the recent advances in research on the CLC chloride channels of the kidney with a special focus on the issues most necessary to understand their physiological roles in vivo, i.e., their intrarenal and cellular localization and their phenotypes of humans and mice that have their loss-of-function mutations.

knockout mice; immunohistochemistry; nephrogenic diabetes insipidus; Bartter’s syndrome; Dent’s disease

SINCE THE INITIAL CLONING of the CLC family, the isolation of CLC-0 from the electric organ of Torpedo marmorata by an expression cloning strategy (15), nine mammalian CLCs have been cloned by using homology-based cloning methods (1, 3, 14, 18, 19, 35, 42, 43, 45, 46). With the exception of CLC-1, a chloride channel specific to the skeletal muscle, all of the other eight CLCs are expressed in the kidney. However, the physiological roles of most of these channels have not yet been established. To determine the in vivo role of each channel, we must first ascertain its immunolocalization in tissues and cells, as well as its functional characteristics. However, specific antibodies to each CLC channel have not been generated with great success. So far, reports on immunolocalization have been published on only three CLCs [CLC-K1 (47, 48), CLC-K2 (48, 49), and CLC-5 (5, 10, 25, 36)] in the kidney. The difficulty in generating antibodies may be due to the high homology between CLC channels, i.e., CLC-K1 and CLC-K2; CLC-3, CLC-4, and CLC-5. On the other hand, functional expression studies have not necessarily given us definitive clues to elucidate the physiological role of CLC channels in vivo because some of them could not be expressed in heterologous expression systems and the results have not been consistent among the researchers (1, 6, 7, 16, 17, 19, 35, 43, 46). Recently, two human genetic approaches have clearly determined important physiological roles of CLC-5 and CLC-Kb in kidney (23, 39). In addition, we recently generated CLC-K1-knockout mice and found that the Clcnk1−/− mice showed nephrogenic diabetes insipidus (27). The impact of these studies has clarified the physiological relevance of CLC chloride channels in the kidney and highlighted the importance of these genetic approaches in characterizing the physiological roles of channels and transporters in this organ.

CLC-K1 AND CLC-K2 CHLORIDE CHANNELS

Molecular Cloning and Intrarenal Localization

CLC-K1 (46) and CLC-K2 (1) were isolated by using a PCR-based cloning strategy from rat kidney. These
chloride channels are highly homologous proteins (~80% amino acid identity), each consisting of 687 amino acids. Later, Kieferle et al. (19) reported the cloning of two human CLC-K channels and tentatively named them CLC-Ka and CLC-Kb. Because the amino acid identity between human CLC-Ka and CLC-Kb was >90%, sequence comparison did not tell us which clone corresponded to K1 or K2 in rats. We also cloned a human CLC-K channel that we identified as a human homolog of rat CLC-K2 on the basis of its localization in the human kidney (44). This clone turned out to be identical with CLC-Kb (19). In other species, Zimniak et al. (51, 52) cloned a rabbit CLC-K channel and designated it as rbCLC-Ka. Very recently, Maulet et al. (28) reported the isolation of the Xenopus laevis CLC-K channel. There are also two CLC-K channels from mice deposited by Zimniak et al. in GenBank (accession nos. AF124847 and AF124848), and we knocked out one of them, AF124848. Although this channel is named the CLC-K channel in the cortical thick ascending limb of Henle’s loop (cTAL), our immunohistochemistry (27) clearly showed that it is a mouse homolog of rat CLC-K1 (see Fig. 1).

Exact intrarenal localization of two CLC-K channels was clearly determined by a recent in situ hybridization study in which CLC-K1- and -K2-specific riboprobes were prepared in the 3’-untranslated regions. This study verified our initial immunohistochemical study on CLC-K1 (47) and also showed that CLC-K1 and CLC-K2 are not colocalized. Immunohistochemistry revealed that CLC-K1 is restricted in the thin ascending limb of Henle’s loop (tAL) and that it is present in both the apical and basolateral plasma membranes (47). The presence of CLC-K1 in both plasma membranes explains why tAL possesses an extraordinarily high chloride permeability (13) and why TAL having CLC-K2 only on the basolateral side shows much less transepithelial chloride permeability. In contrast to the restricted localization of CLC-K1, CLC-K2 showed a relatively broader expression pattern in distal nephrons (50). In situ hybridization revealed that CLC-K2 is expressed abundantly in the distal tubules, connecting tubules, and cortical collecting ducts (50). There is also moderate expression in the medullary TAL (mTAL). As for the cellular localization of CLC-K2, there has been no report using a CLC-K2-specific antibody. In the study by Vandewalle et al. (48), an antibody recognizing both CLC-K1 and CLC-K2 stained the basolateral plasma membranes of the distal nephrons, including inner medullary collecting ducts. Winters et al. (49) reported that rbCLC-Ka was present in the basolateral plasma membranes of mTAL and in the cytoplasm of intercalated cells of collecting ducts. Recently, we found in an analysis of CLC-K1-knockout mice that our antiserum specific to rat CLC-K1 recognized both CLC-K1 and CLC-K2 in mice (K. Kobayashi and S. Uchida, unpublished observations; see Fig. 1). Accordingly, we could observe CLC-K2 immunohistochemistry in the section of Clcnk1−/− mice. Specifically, we could observe a basolateral staining of CLC-K2 in the mTAL, distal tubules, and connecting tubules in that section. There was no immunoreactivity in the collecting ducts in the inner medulla. These results were highly consistent with our in situ hybridization study in rats (50). Taken in sum, the findings from all these studies suggest that CLC-K2 is a basolateral chloride channel in the nephron segment where sodium-dependent chloride transporters (furosemide-sensitive Na-K-2Cl cotransporter and thiazide-sensitive Na-Cl cotransporter) are present in the apical plasma membranes. This suggested a role of CLC-K2 as a route for vectorial transepithelial chloride transport (reabsorption). Figure 2 shows the localization of various CLC channels along the nephron.
Nephrogenic Diabetes Insipidus in Clcnk1−/− Mice and Bartter’s Syndrome in Patients with CLCNKB Mutations

Before we generated CLC-K1-knockout mice, Simon et al. (39) reported mutations of the CLCNKB gene in patients with Bartter’s syndrome. As mentioned above, localization of CLC-K2 in the kidney suggested the role of CLC-K2 in chloride reabsorption in distal nephrons. Reduction of chloride reabsorption in these nephron segments was known to cause hypovolemic hypokalemic alkalosis, and Simon et al. (40, 41) actually found mutations of genes encoding furosemide-sensitive Na-K-2Cl cotransporter and thiazide-sensitive Na-Cl cotransporter in patients with Bartter’s and its variant, Gitelman’s syndrome, respectively. Accordingly, the CLCNKB gene (human gene of CLC-K2) became the third gene with mutations known to cause Bartter’s syndrome. This study clearly demonstrated the anticipated role of CLC-K2 in the kidney, i.e., as a route for chloride reabsorption to maintain extracellular volume. More direct evidence will be obtained by a microperfusion study of TAL from the Clcnk2−/− mice.

In contrast to CLC-K2, CLC-K1 is present only in the tAL. Considering its structural similarity to CLC-K2 and its plasma membrane localization, there was no doubt that CLC-K1 was also involved in the transepithelial chloride transport in tAL. However, no loss-of-function mutation was found in the CLCNKA gene (human CLC-K1 gene) in patients with Bartter’s syndrome (39). This suggested that chloride transport in the tAL may not be involved in the regulation of chloride balance in the extracellular space. Rather, CLC-K1 may well be involved in urinary concentrating mechanisms as an important component of countercurrent systems. To directly verify this possibility, we generated CLC-K1-knockout mice (27). Immunohistochemistry revealed the selective deletion of the CLC-K1 channel in the tAL, i.e., CLC-K1 (Fig. 1). In a microperfusion study of the tAL of Clcnk1−/− mice, we could clearly conclude that the transepithelial chloride transport in the tAL is mediated by CLC-K1 (27). As expected, knockout of chloride transport in the tAL resulted in polyuria that was insensitive to deamino-Cys1, D-Arg8 vasopressin (dDAVP) administration, i.e., nephrogenic diabetes insipidus (Fig. 3). We recently confirmed that this polyuria was the result of water diuresis, not solute diuresis (N. Akizuki and S. Uchida, unpublished observations). This indicated that, unlike the mutation of CLC-K2 in humans, the change of transpithelial chloride transport in the tAL did not affect chloride clearance. On the other hand, direct measurement of inner medullary osmolarity was measured.

![CLC-K1, CLC-K2, and CLC-5](image-url)
CLC-5 encodes a 746-amino acid protein and forms a subbranch with CLC-3 (17, 18) and CLC-4 (14). Although these three CLC channels possess only ~30% amino acid identity with other CLC branches, they share ~80% identity amongst themselves, and this has made the generation of specific antibodies difficult. In rats, CLC-5 is expressed abundantly in kidney, and much lower expression is observed in the colon, brain, and liver (35, 43). CLC-3 expression has been observed in various rat tissues including the kidney (18). Rat CLC-4 is expressed in the skeletal muscle, heart, kidney, brain, and liver (14). Thus all three of these channels are expressed in the kidney. Information on the localization of these CLC channels in the kidney was scant until the appearance of four recent papers on the intrarenal and cellular localization of CLC-5 (5, 10, 25, 36). Still, there has been no report on the localization of CLC-4 in the kidney, and only one in situ hybridization study revealed that CLC-3 is expressed in the intercalated cells (type B) of collecting ducts (32). As for CLC-5 localization, Günther et al. (10) first reported rat CLC-5 immunolocalization in kidney by using polyclonal antibody raised against a synthetic peptide as an antigen. CLC-5 was present in the endocytic vesicles of S1, S2, and S3 segments of the proximal tubules in this study. It was concentrated below microvilli of the brush border and colocalized with \(\alpha\)-ATPase. In intercalated cells of collecting ducts, it again localizes to apical intracellular vesicles and colocalizes with \(\alpha\)-ATPase in \(\alpha\)-intercalated cells. Very recently, we reported mouse CLC-5 immunolocalization using a monoclonal antibody raised against a synthetic peptide as an antigen (36). The results were basically similar to those on rat CLC-5 reported by Günther et al. (10). On the other hand, Luyckx et al. (25) reported CLC-5 staining in the S3 segment of proximal tubules and mTAL but not in the intercalated cells. They raised antisera against a CLC-5 fusion protein and immunoabsorbed CLC-3 and CLC-4 reactivity to generate CLC-5-specific antiserum. The discrepancy between this study and the former two cannot be explained merely by species difference or differences in methodology. Luyckx et al. claimed that the CLC-5 staining reported by Günther et al. (10) could be staining of CLC-3 and/or CLC-4, instead of CLC-5. Determination of specificity of polyclonal antibodies is sometimes very difficult, especially when closely related molecules are present. The fact that our monoclonal data corroborated the report by Günther but not that by Luyckx et al. (25) suggested that the staining of the subapical vesicles in proximal tubule and intercalated cells may not be the staining of CLC-3 and/or CLC-4, but rather of CLC-5 (Fig. 2). Another CLC-5 immuno-histochemical study by Devuyst et al. (5) did not convincingly support either of these discrepant results. They showed human CLC-5 localization in proximal tubules, TAL, and intercalated cells of collecting ducts by using an immunoabsorbed CLC-5-specific polyclonal antibody raised against a synthetic peptide corresponding to CLC-5 extracellular domains (5). The staining in the proximal tubules and intercalated cells appeared broadly cytoplasmic and did not colocalize with \(\alpha\)-ATPase. Taken together, these findings do not yet provide a definitive answer on the localization of CLC-3, CLC-4, and CLC-5 in the kidney. Immunostaining of kidney sections of patients or animals deficient in each CLC channel may be the best way to determine the specificity of antibodies.

**CLCN5 Mutations in Human Kidney Diseases**

Mutations in CLCN5 (the human gene of CLC-5) were found in three disorders associated with hypercalcemic nephrolithiasis, i.e., Dent’s disease, X-linked recessive nephrolithiasis, and X-linked recessive hypophosphatemic rickets (22, 23, 34). Later, several groups including our own identified CLCN5 mutations in Japanese patients with low-molecular-weight proteinuria (LMWP) (2, 12, 24, 30, 31), a disease first reported about 20 years ago in children. Because there is an annual urinary screening system for children in Japan, asymptomatic children with LMWP can often be found. As a result, we could find the mutations of the CLCN5 gene in patients with LMWP as a sole phenotype (30). It is not known whether these patients will remain asymptomatic or will develop various symptoms other than LMWP in the future. In any case, the existence of patients having the CLCN5 gene mutations who showed only LMWP suggested that LMWP is a common and essential manifestation of the dysfunction of CLC-5. It is well known that low-molecular-weight proteins are reabsorbed in the proximal tubules by endocytotic processes. Accordingly, the localization of CLC-5 in the endosomes of proximal tubules and the colocalization with \(\alpha\)-ATPase shown by Günther et al. (10) and our own group (36) neatly explain the pathogenesis of this essential phenotype of CLC-5 disorder. However, there are still several unresolved problems in the pathogenesis of various phenotypes in Dent’s disease and X-linked recessive nephrolithiasis if the primary defect in the CLC-5 channel disorder is assumed to be a defect of endocytosis in the proximal tubules. For example, CLC-5 current expressed on heterologous expression systems (43) did not fit with the expected characteristics of chloride channels in endo-
somess. Even if CLC-5 is assumed to be present in mTAL, an important site for calcium reabsorption, it is still difficult to explain how an intracellular chloride channel can be involved in calcium transport in mTAL. Very recently, Silva et al. (38) reported that parathyroid hormone (PTH) modulated the expression of CLC-5 in the kidney cortex but that neither 1α,25(OH)₂ vitamin D₃ nor PTH regulated CLC-5 expression in the medulla. This suggested that CLC-5 could be involved in calcium homeostasis, but the exact mechanism is still obscure. To solve these problems, a CLC-5-deficient animal model may be necessary. Recently, Luyckx et al. (26) reported a mouse model of reduced CLC-5 expression investigated by a ribozyme approach. Surprisingly, the transgenic ribozyme-expressing animals showed no obvious phenotype except for a slight hypercalciuria. LMWP was not mentioned in their study. Thus we have to wait for the real CLC-5-knockout mice to answer the above unresolved problems.

The physiological roles of CLC-3 and CLC-4 in vivo remain to be determined. Duan et al. (6) reported CLC-3 as a volume-regulated chloride channel. Considering their structural similarity to CLC-5, however, CLC-3 and CLC-4 may also be intracellular chloride channels. To clarify their physiological roles, their exact cellular localization and the use of knockout animals or cells will first be necessary.

OTHER CLC CHLORIDE CHANNELS

There has been no mention thus far in this report of three other mammalian CLC chloride channels, CLC-2 (32), CLC-6 (4, 20), and CLC-7 (20). Although their functions and tissue distributions have been partially characterized, their physiological roles in vivo still remain somewhat obscure because of the lack of information on exact cellular localization. Yeast has one CLC gene, GEFI, which is involved in ionic homeostasis of intracellular organelles (8, 9). CLC-3 and CLC-5 homolog were also cloned from A6 X. laevis renal cells (21) and gills of Oreochromis mossambicus (29). In addition, Arabidopsis thaliana is known to have six CLC chloride channels, one of which (AtCLC-d) functions as an intracellular chloride channel (11). Recently, nematode was found to have six CLC chloride channels (37). Petalcorin et al. (33) reported that one (clh-1) of the null mutations caused a significantly wider body and that this condition was restored to normal by high osmolarity in the culture medium. The authors speculated that clh-1 is somehow involved in the osmoregulation.

FUTURE DIRECTIONS

Recent human genetic studies (23, 39) have determined the in vivo roles of CLC-K2 and CLC-5 in the kidney. Research on ion channels can benefit tremendously from the discovery of channel diseases in humans. Naturally occurring mutations causing diseases show us regions of functional importance in the channels and give us important information on the structure-function relationship of channel proteins. However, it is not always possible to find mutations of a gene of interest in humans. Accordingly, a generation of knockout animals is an important strategy in the study of the physiological role of a gene of interest. In addition, as in the case of CLC-5, a mouse model must be generated to understand the exact mechanisms of a disease even after loss-of-function mutations were found in humans. There still remain CLC channels with unknown in vivo functions, for which knockout mice must be generated and analyzed in the future. Cellular localization of a channel protein and its regulation (intracellular sorting mechanisms) are also important issues in understanding channel physiology. In research on mammalian CLC chloride channels, cellular localization at the electron microscopy level has only been studied with regard to CLC-K1 and CLC-5, and there has not been a report on sorting mechanisms within cells. More studies on this issue should be performed in the future because functions of CLC chloride channels could be regulated at the level of intracellular trafficking of channel proteins. It is also not known whether CLC chloride channels are associated with a variety of molecules to achieve the physiological function of the ion channels. The failure to express some CLC chloride channels in heterologous expression systems suggests this possibility. Identification of such associated proteins could also help to clarify the unknown physiological role of CLC channels.

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REFERENCES


