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Interaction of neuron specific K^+-Cl^- cotransporter, KCC2, with brain-type creatine kinase

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Abstract **γ-Aminobutyric acid, a major inhibitory neurotransmitter within the adult central nervous system, is also known to be excitatory at early developmental stages due to the elevated intracellular Cl^- concentration. This functional change is primarily attributable to a K^+-Cl^- cotransporter, KCC2, the expression of which is developmentally regulated in neurons. However, little detail information is available concerning the intracellular regulation of KCC2 function. Here, we identify an interaction between KCC2 and brain-type creatine kinase by means of yeast two-hybrid screening. This interaction, which was also detected in cultured cells and brain extracts, might contribute to KCC2-mediated modulation of Cl^- homeostasis.**

Key words: KCC2; Creatine kinase; Two-hybrid; ATP; Cl^- homeostasis

1. Introduction

γ-Aminobutyric acid (GABA) is well known to be a major inhibitory neurotransmitter within the adult central nervous system, but recently it has also been found to function as an excitatory neurotransmitter both during the developmental stage and during periods of neuronal injury [1–3]. Whether it is inhibitory or excitatory would seem to be determined by the intracellular Cl^- concentration ($[Cl^-]i$) [1,4,5]. Thus, in immature or injured neurons, the relatively high $[Cl^-]i$ renders the Cl^- equilibrium potential (E_{Cl}) positive to the resting membrane potential, so that activation of $GABA_A$ receptor channels results in an outward flux of Cl^- and a consequent depolarization of the neuron. In contrast, in mature neurons E_{Cl} is so negative to the resting membrane potential, due to the low $[Cl^-]i$, that activation of the above channels leads to an influx of Cl^- and a consequent neuronal hyperpolarization.

Electroneutral transporters for Cl^- play important roles in determining the resting $[Cl^-]i$, and a neuron-specific K^+-Cl^- cotransporter, KCC2 [6], is involved in the developmental regulation of $[Cl^-]i$ in neurons [3]. This cotransporter extrudes K^+ and Cl^- out of the cells, so reducing $[Cl^-]i$, and its expression is upregulated during development [7–9]. However, it is downregulated after neuronal injury, resulting in an elevated $[Cl^-]i$ [10,11]. Thus, KCC2 is implicated in the developmentally regulated bidirectional responses to GABA. In addition, it has been reported that KCC2 is localized at dendritic spines [12], and colocalized with either GABA receptors [13] or gephyrin, which clusterizes GABA and glycine receptors [14], indicating a crucial role in the control of the responses to GABA and glycine. However, we have as yet few details about the intracellular regulation of KCC2, except the inference that some protein kinases modulate its function [15,16]. This gave us the idea that unidentified protein(s) may affect the function of KCC2 by interacting with molecules physically close to KCC2. In this report, we describe a yeast two-hybrid screening study using the C-terminal cytoplasmic region of KCC2 as bait, and isolated brain-type creatine kinase (CKB), an ATP-generating enzyme.

2. Materials and methods

2.1. Plasmid constructs

The cDNAs of CKB, KCC1, and Na^+-K^+-Cl^- cotransporter 1 (NKCC1) were obtained by means of the polymerase chain reaction (PCR), using adult rat brain cDNA as a template. The full-length KCC2 cDNA (kindly provided by Dr. J.A. Payne) was amplified by PCR and subcloned into pCMV-Tag2B (Stratagene). PCR products encoding 645–1116 amino acids (aa), 645–1114 aa, 645–1048 aa, and 645–914 aa of KCC2, 665–1085 aa of KCC1, and 751–1203 aa of NKCC1 were subcloned into pBTM116 (kindly provided by Dr. M. Hagiwara). The PCR fragment of CKB was cloned into pcDNA3 (Invitrogen) expressing a hemagglutinin (HA) tag. All clones derived by PCR were verified by DNA sequencing.

2.2. Two-hybrid assays

Two-hybrid screening assays were performed as described previously [17]. Briefly, for the present purposes yeast strain L40 was co-transformed with pBTM116-KCC2 (645–1116 aa) and a rat brain cDNA library constructed in pACTII (Clontech). Positive clones on synthetic complete plates lacking tryptophan, leucine, and histidine were further analyzed.

2.3. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. Transfection was performed with the aid of Lipofectamine (Invitrogen) according to the manufacturer's protocol.

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Abbreviations: GABA, γ-aminobutyric acid; $[Cl^-]i$, concentration of intracellular Cl^-; E_{Cl}, Cl^- equilibrium potential; KCC, K^+-Cl^- cotransporter; CKB, brain-type creatine kinase; NKCC, Na^+-K^+-Cl^- cotransporter; PCR, polymerase chain reaction; aa, amino acids; HA, hemagglutinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; CK, creatine kinase; CKM, muscle-type creatine kinase; PCr, phosphocreatine

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2.4. Coimmunoprecipitation

To provide culture cells, 1×10^6 cells growing on 10-cm Petri dishes were transfected with appropriate plasmids for 48 h, then lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, and protease inhibitor). After centrifugation at 12 000 rpm at 4°C for 30 min, 250 μg of the supernatants were incubated with 5 μg of anti-Flag M2 antibody (Sigma) and protein G (Amersham Pharmacia Biotech) for 2 h at 4°C. After washing three times with wash buffer (lysis buffer without protease inhibitor), the pellets were mixed with 1×Laemmli sample buffer, and boiled. They were then centrifuged, and the supernatants were analyzed by immunoblotting using anti-HA (1:1000; MBL) and anti-KCC2 (1:2500; Upstate Biotechnology) antibodies.

To make brain extracts, rat whole brains were homogenized, then lysed in lysis buffer. The subsequent protocol was as described above except for the use of anti-KCC2 antibody, rabbit IgG (Sigma), or anti-NKCC1 monoclonal antibody (T4; Departmental Studies Hybridoma Bank, Iowa City, IA, USA) instead of anti-Flag M2 antibody for the immunoprecipitation, and anti-CKB antibody (1:500; Santa Cruz) instead of anti-HA antibody for the immunoblotting.

2.5. Immunoblotting

Samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by electrotransfer onto polyvinylidene difluoride membranes. For visualization, blots were probed with appropriate primary antibodies, then detected using horseradish peroxidase-conjugated secondary antibodies (1:1000; New England BioLabs) and an ECL kit (Amersham Pharmacia Biotech).

2.6. Immunofluorescence staining

Transfected cells on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeabilization in PBS containing 0.2% Triton X-100. The cells were then incubated with anti-Flag M2 (1:1000) and anti-HA (1:1000) antibodies, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit (1:100; Zymed Laboratories) and Texas red-conjugated anti-mouse (1:100; Molecular Probes) antibodies. For DNA staining, coverslips were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Fluorescent images were analyzed by a fluorescence microscopy.

3. Results

3.1. Isolation of a KCC2-interacting protein using yeast two-hybrid screening

When we screened 1×10^6 transformants of the rat brain cDNA library using as bait the C-terminal cytoplasmic region of KCC2 (645–1116 aa), we obtained 60 positive clones. After confirmation and partial sequencing, 22 clones were found to encode CKB (Fig. 1A). The common region of overlap shared by all the CKB spanned 273–381 aa.

To identify the precise region of KCC2 required for the interaction with CKB, we prepared various truncated constructs of the C-terminal cytoplasmic region of KCC2, and examined the interaction with CKB using the two-hybrid assay. CKB interacted with 645–1116 aa and 645–1114 aa, but not with 645–1048 aa or 645–914 aa, of KCC2 (Fig. 1B), indicating that the 1049–1114 aa region of KCC2 is required for the interaction.

3.2. Interaction of KCC1, but not NKCC1, in yeast two-hybrid assay

Alignments of amino acid sequences showed that the 1049–1114 aa region of KCC2 is highly homologous to the corresponding regions of KCC1, KCC3, and KCC4, which are the other members of the K^+-Cl^- cotransporter family, but not to that of NKCC1, which is one of the cation-Cl^- cotransporter superfamily (Fig. 2A). We therefore examined whether CKB

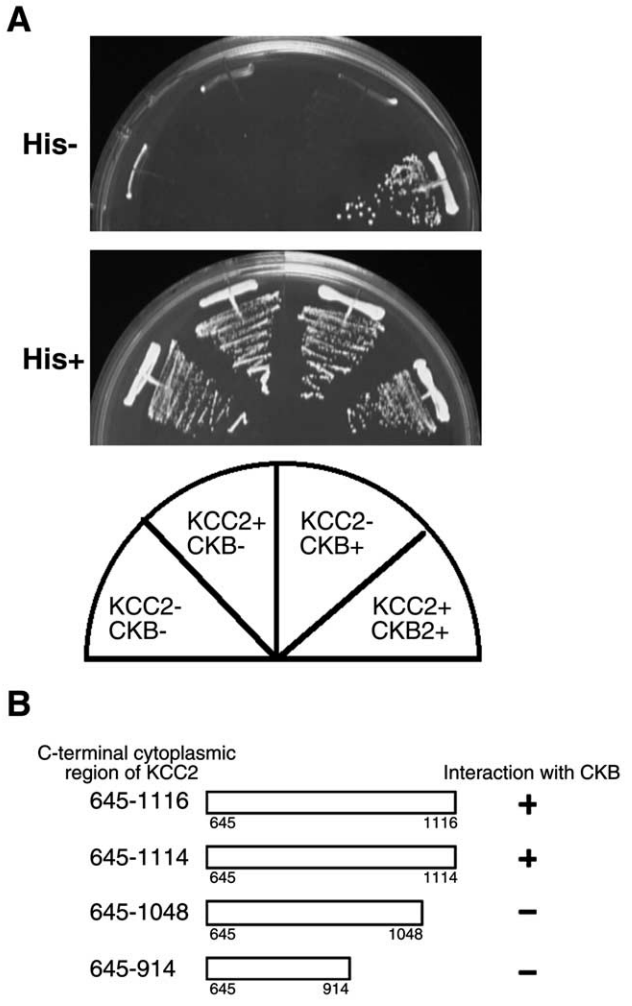

Fig. 1. Interaction of CKB with KCC2 in yeast two-hybrid assay. A: KCC2+ indicates lexA fused to the C-terminal cytoplasmic region of KCC2 used as bait, while KCC2− indicates lexA alone in the vector pBTM116. CKB (260–381 aa) was either fused (CKB+) to the Gal4p activation domain in the vector pACTII, or not fused (CKB−). The transformants were selected on Trp− Leu− plates (His+), and the interactants were subsequently selected on Trp− Leu− His− plate (His−). B: Schematic diagram showing the results, from the yeast two-hybrid assay, concerning the interaction between CKB and various deletion constructs of KCC2. Numbers refer to amino acid positions in rat KCC2.

interacts with other Cl^- transporters, such as KCC1 and NKCC1, the expressions of which have been identified in brain [18]. CKB was found to associate with the C-terminal cytoplasmic region of KCC1, but not with that of NKCC1 (Fig. 2B).

3.3. Interaction of KCC2 with CKB in culture cells

To determine whether CKB associates with KCC2 in vivo, we performed coimmunoprecipitation assays. COS-7 cells were transiently transfected with or without plasmids expressing full-length HA-CKB and/or full-length Flag-KCC2. After immunoprecipitation using anti-Flag M2 antibody, HA-CKB was detected in a cell lysate containing both HA-CKB and Flag-KCC2 by the use of anti-HA antibody, but not in the others (Fig. 3A).

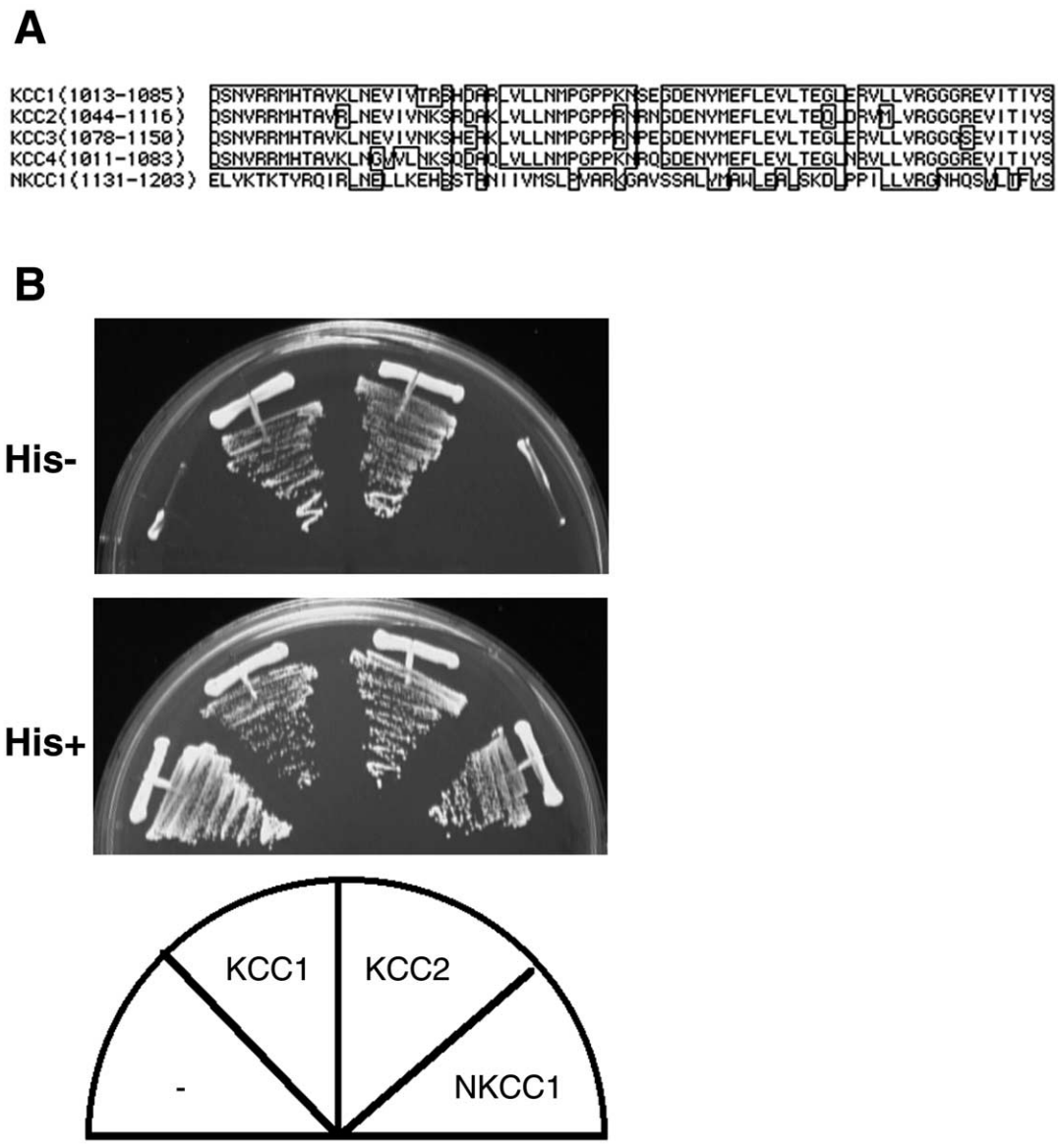

Fig. 2. Interaction of CKB with various Cl^- transporters in yeast two-hybrid assay. A: C-terminal amino acid sequences for representative rat (KCC2, KCC1, and NKCC1) or human (KCC3 and KCC4) Cl^- transporters. Identical amino acid residues are highlighted by boxes. B: The C-terminal cytoplasmic region of the indicated transporter was either fused to the Gal4p activation domain in the vector pACTII, or not fused (−). The transformants with lexA-fused CKB (260–381 aa) were selected as described in Fig. 1A.

3.4. Subcellular distributions of KCC2 and CKB in culture cells

Next, we examined whether KCC2 colocalized with CKB in transfected COS-7 cells. Immunofluorescence analysis showed that CKB was distributed mainly throughout the cytoplasm. KCC2 was located mostly in the perinuclear region, but also in the cytoplasm (Fig. 3B). These observations suggest that KCC2 and CKB colocalized to some extent. Essentially, the same experiment was then performed using HEK293 cells, and similar results were obtained (data not shown).

3.5. Interaction of KCC2 with CKB in brain extract

To explore whether KCC2 and CKB can associate under physiological conditions, we carried out immunoprecipitations using rat brain extracts. Immunoprecipitation by anti-KCC2 antibody resulted in a coprecipitation of CKB in the brain extract, whereas CKB was not coprecipitated with a control rabbit IgG or anti-NKCC1 antibody (Fig. 4). This result indicates that endogenous KCC2 and CKB associate with each other.

4. Discussion

Here, we used yeast two-hybrid screening to identify CKB as a binding partner of KCC2, one of the K^+-Cl^- cotransporters belonging to the electroneutral cation-Cl^- cotransporter superfamily. Our deletion analysis showed that 1048–1114 aa of KCC2 and 273–381 aa of CKB are important for the interaction. Furthermore, KCC2 and CKB were found to interact in transfected COS-7 cells and in adult rat brain, suggesting that CKB truly is a partner of KCC2 in vivo.

In higher vertebrates, four creatine kinase (CK) isoenzymes are present: namely, brain (CKB), muscle (CKM), and two mitochondrial CKs [19]. CKB and CKM are functionally active as dimers in three isozymic forms: BB (the predominant

form in brain), MM (in skeletal muscle) and MB (in cardiac muscle). CKs regenerate ATP by catalyzing the exchange of high-energy phosphates between γ-ATP and phosphocreatine (PCr) according to: $MgATP^{2-}$+creatine ↔ $MgADP^{-}$+PCr^{2-}+H^{+} [19]. CKB, which is expressed in a number of tissues, is especially abundant in brain [20], in which most

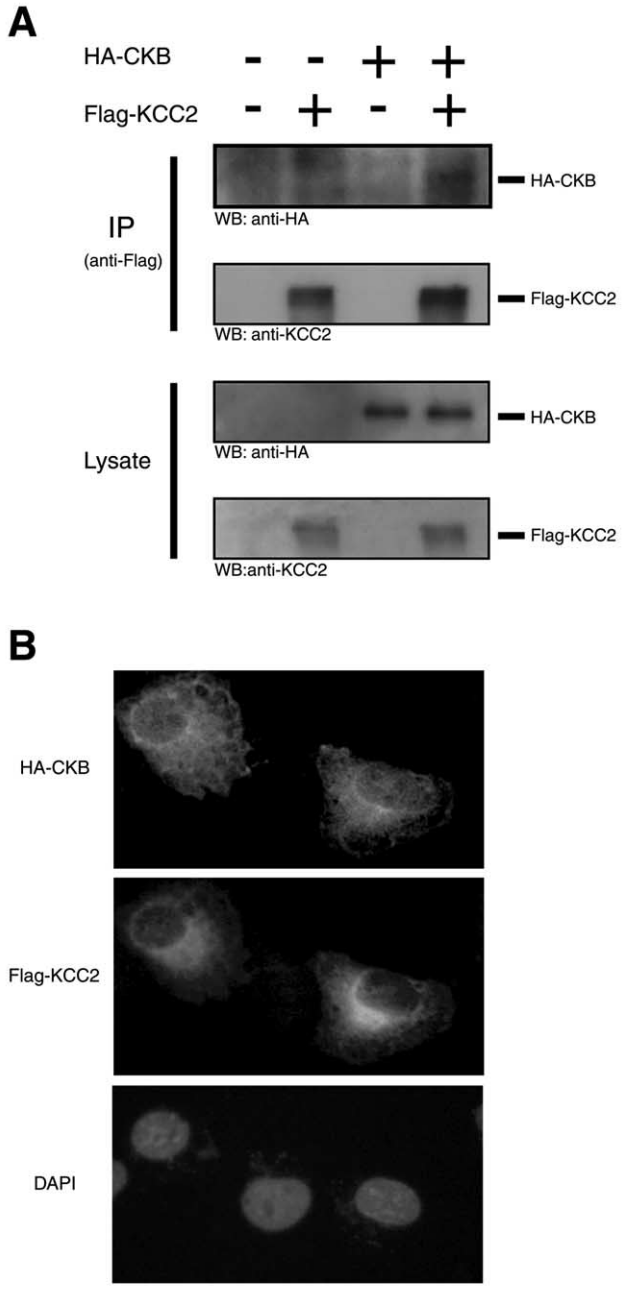

Fig. 3. Coimmunoprecipitation and colocalization of CKB with KCC2. A: HA-CKB was transiently transfected into COS-7 cells with or without Flag-KCC2. The cells were lysed, and then immunoprecipitation was performed using anti-Flag monoclonal antibody. The lysates and immunoprecipitates were analyzed by SDS–PAGE and immunoblotted using anti-KCC2 and anti-HA polyclonal antibodies. B: COS-7 cells were transiently transfected with expression plasmids for HA-CKB and Flag-KCC2, and an immunofluorescence analysis was performed using anti-HA polyclonal (top) or anti-Flag monoclonal (middle) antibodies. For DNA staining, the cells were incubated with DAPI (bottom).

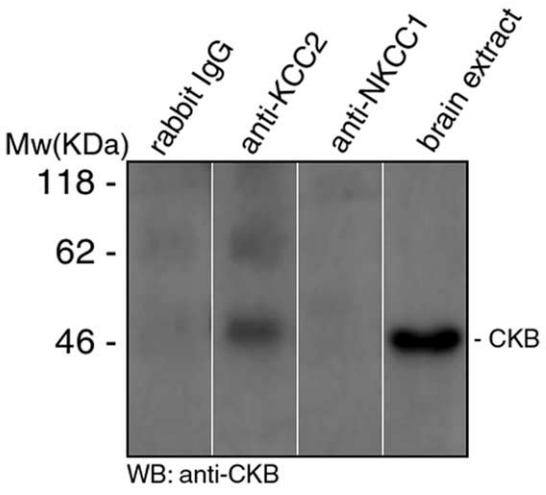

Fig. 4. Interaction of CKB with KCC2 in rat brain extracts. Solubilized extracts from adult rat brain were subjected to immunoprecipitation analysis using rabbit IgG, anti-KCC2 antibody, or anti-NKCC1 antibody. Extracts (5 μg) and immunoprecipitates were analyzed by SDS–PAGE and immunoblotted using anti-CKB polyclonal antibodies. Precipitation of KCC2 or NKCC1 was detected using anti-KCC2 or anti-NKCC1 antibody, respectively (data not shown).

CKB has been shown to be cytosolic, although a number of reactions requiring CKB are membrane-associated. Indeed, CKB has been found to bind to a membrane-associated thrombin receptor, PAR-1, leading to functional activation [21]. In addition, CKs have been reported to interact physically with the ATP-sensitive K^{+} channel [22] and to localize to acetylcholine receptor-rich membranes [23]. Thus, membrane-associated CKs are considered to play an important role in regenerating the ATP necessary for the ion-driving force, although the question as to the precise identity of the targets has not been fully addressed.

In neuronal cells, CKB is located in synaptic regions [24], in which KCC2 is also localized [14]. Moreover, Na^{+}-K^{+} ATPase preferentially localizes to dendrites [25] and is considered to be functionally coupled either with KCC2 or, independently, with CKB [3,26]. From this point of view, the notion of an interaction between KCC2 and CKB would seem to be reliable. Since CKB also seems to be associated with the ubiquitously expressed KCC1, we employed the two-hybrid assay to examine the interactions between CKM and KCCs in the hope of revealing whether interactions between KCCs and CKs are common phenomena. However, we could not detect any interaction between KCCs and CKM (data not shown).

The functional significance of the interaction between KCC2 and CKB is not clear, and how the intracellular ATP level might contribute to KCC2 function is still a matter for discussion. Since an ATP is necessary for activation of Na^{+}-K^{+} ATPase, leading to a driving force for KCC, ATP should enhance the function of KCC2. Indeed, some reports have affirmed an ATP-induced KCC activation [27,28], although contrariwise a decrease in the ATP level has also been reported to activate KCC [29]. In another possible scheme, CKB may provide ATP to other molecules, such as kinases, that are physically close to KCC2, so helping to maintain membrane Cl^{-} homeostasis. Indeed, a broad-spectrum protein kinase inhibitor, staurosporine, stimulates KCC activity

while protein tyrosine kinases enhance KCC2 functional activity [16,30]. In this concern, aside from the provision of ATP, CKB might phosphorylate KCC2 to change its function, because CKB possesses autophosphorylation activity [31]. Although these issues require further detailed exploration, the findings in this report do at least provide an insight into the relationship between KCCs and ATP.

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References

[1] Ben-Ari, Y. (2002) Nat. Rev. Neurosci. 3, 728–739.
[2] Owens, D.F. and Kriegstein, A.R. (2002) Nat. Rev. Neurosci. 3, 715–727.
[3] Payne, J.A., Rivera, C., Voipio, J. and Kaila, K. (2003) Trends Neurosci. 26, 199–206.
[4] Ben-Ari, Y., Cherubini, E., Corradetti, R. and Gaiarsa, J.L. (1989) J. Physiol. 416, 303–325.
[5] Cherubini, E., Gaiarsia, J.L. and Ben-Ari, Y. (1991) Trends Neurosci. 14, 515–519.
[6] Payne, J.A., Stevenson, J. and Donaldson, L. (1996) J. Biol. Chem. 271, 16245–16252.
[7] Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M. and Kaila, K. (1999) Nature 397, 251–255.
[8] Lu, J., Karadsheh, M. and Delpire, E. (1999) J. Neurobiol. 39, 558–568.
[9] Ganguly, K., Schinder, A.F., Wong, S.T. and Poo, M.-M. (2001) Cell 105, 521–532.
[10] Toyoda, H., Ohno, K., Yamada, J., Ikeda, M., Okabe, A., Sato, K., Hashimoto, K. and Fukuda, A. (2003) J. Neurophysiol. 89, 1353–1362.
[11] Coull, J.A.M., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sik, A., De Koninck, P. and De Koninck, Y. (2003) Nature 424, 938–942.
[12] Hübner, C.A., Stein, V., Hermans-Borgmeyer, I., Meyer, T., Ballanyi, K. and Jentsch, T.J. (2001) Neuron 30, 515–524.
[13] Williams, J.R., Sharp, J.W., Kumari, V.G., Wilson, M. and Payne, J.A. (1999) J. Biol. Chem. 274, 12656–12664.
[14] Gulyás, A.I., Sik, A., Payne, J.A., Kaila, K. and Freund, T.F. (2001) Eur. J. Neurosci. 13, 2205–2217.
[15] Strange, K., Singer, T.D., Morrison, R. and Delpire, E. (2000) Am. J. Physiol. 279, C860–C867.
[16] Kelsch, W., Hormuzdi, S., Staube, E., Lewen, A., Monyer, H. and Misgeld, U. (2001) J. Neurosci. 21, 8339–8347.
[17] Inoue, K., Mizuno, T., Wada, K. and Hagiwara, M. (2000) J. Biol. Chem. 275, 32793–32799.
[18] Delpire, E. and Mount, D.B. (2002) Annu. Rev. Physiol. 64, 803–843.
[19] Wyss, M. and Kaddurah-Daouk, R. (2000) Physiol. Rev. 80, 1107–1213.
[20] Sistermans, E.A., de Kok, Y.J.M., Peters, W., Ginsel, L.A., Jap, P.H.K. and Wieringa, B. (1995) Cell Tissue Res. 280, 435–446.
[21] Mahajan, V.B., Pai, K.S., Lau, A. and Cunningham, D.D. (2000) Proc. Natl. Acad. Sci. USA 97, 12062–12067.
[22] Crawford, R.M., Ranki, H.J., Botting, C.H., Budas, G.R. and Jovanovic, A. (2002) FASEB J. 16, 102–104.
[23] Barrantes, F., Mieskes, G. and Wallimann, T. (1983) FEBS Lett. 152, 270–276.
[24] Friedman, D.L. and Roberts, R. (1994) J. Comp. Neurol. 343, 500–511.
[25] Brines, M.L., Tabuteau, H., Sundaresan, S., Kim, J., de Spencer, D.D. and Lanerolle, N. (1995) Epilepsia 36, 371–383.
[26] Blum, H., Balschi, J.A. and Johnson Jr., R.G. (1991) J. Biol. Chem. 266, 10254–10259.
[27] Lauf, P.K. (1983) Am. J. Physiol. 245, C445–C448.
[28] Lauf, P.K., Bauer, J., Adragna, N.C., Fujise, H., Martin, A., Zade-Oppen, M., Ryu, K.H. and Delpire, E. (1992) Am. J. Physiol. 263, C917–C932.
[29] Ortiz-Carranza, O., Adregna, N.C. and Lauf, P.K. (1996) Am. J. Physiol. 271, C1049–C1058.
[30] Shen, M.-R., Chou, C.-Y., Hsu, K.-F., Liu, H.-S., Dunham, P.B., Holtzman, E.J. and Ellory, J.C. (2001) Proc. Natl. Acad. Sci. USA 98, 14714–14719.
[31] Hemmer, W., Furter-Graves, E.M., Frank, G., Wallimann, T. and Furter, R. (1995) Biochim. Biophys. Acta 1251, 81–90.