Direct Protein Kinase C-dependent Phosphorylation Regulates the Cell Surface Stability and Activity of the Potassium Chloride Cotransporter KCC2

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The potassium chloride cotransporter KCC2 plays a major role in the maintenance of transmembrane chloride potential in mature neurons; thus KCC2 activity is critical for hyperpolarizing membrane currents generated upon the activation of γ-aminobutyric acid type A and glycine (Gly) receptors that underlie fast synaptic inhibition in the adult central nervous system. However, to date an understanding of the cellular mechanism that neurons use to modulate the functional expression of KCC2 remains rudimentary. Using Escherichia coli expression coupled with in vitro kinase assays, we first established that protein kinase C (PKC) can directly phosphorylate serine 940 (Ser940) within the C-terminal cytoplasmic domain of KCC2. We further demonstrated that Ser940 is the major site for PKC-dependent phosphorylation for full-length KCC2 molecules when expressed in HEK-293 cells. Phosphorylation of Ser940 increased the cell surface stability of KCC2 in this system by decreasing its rate of internalization from the plasma membrane. Coincident phosphorylation of Ser940 increased the rate of ion transport by KCC2. It was further evident that phosphorylation of endogenous KCC2 in cultured hippocampal neurons is regulated by PKC-dependent activity. Moreover, in keeping with our recombinant studies, enhancing PKC-dependent phosphorylation increased the targeting of KCC2 to the neuronal cell surface. Our studies thus suggest that PKC-dependent phosphorylation of KCC2 may play a central role in modulating both the functional expression of this critical transporter in the brain and the strength of synaptic inhibition.

Cation-chloride cotransporters (CCC) regulate Cl− homeostasis in cells and the generation of transmembrane chloride gradients (1). Adult mammalian neurons maintain low intracellular Cl− concentrations, which arise principally from the activity of the potassium chloride cotransporter-2 (KCC2). The maintenance of such low levels of intracellular Cl− ions is responsible for hyperpolarizing Cl− currents upon activation of GABA A and Gly receptors, which are responsible for fast synaptic inhibition in the adult central nervous system (2–5). Molecular studies have demonstrated that KCC2 is a member of a CCC superfamily and that these transporters are composed of 12-transmembrane domains with N- and C-terminal cytoplasmic domains (2, 6, 7). KCC2 is expressed exclusively in neurons throughout the adult brain. Developmentally KCC2 is first detected around 10 days in vitro in cultured rat neurons, which is coincident with the emergence of hyperpolarizing GABA A receptor-mediated Cl− currents (4, 8). Gene knock-out of KCC2 has revealed that ablating the expression of this protein results in early postnatal death. Neurons derived from these animals exhibit compromised GABA A receptor-mediated synaptic inhibition (9).

Under pathological conditions such as epilepsy or ischemic brain injury, deficits in the expression of KCC2 are evident together with decreased efficacy of GABAergic inhibition and with the emergence of depolarizing GABA A receptor-mediated currents that reflect decreased neuronal Cl− extrusion (10). These changes in functional expression are believed in part to be transcriptional (11, 12), but post-translational modification of KCC2 is also likely to be of central importance. Intriguingly the activity of a number of protein kinases, including WNK3, WNK4, brain-type creatine kinase, TrkB receptors, tyrosine kinases, and PKC, have all been reported to influence KCC2 activity (13). However, it remains to be established whether these varying kinase activities actually directly regulate KCC2 phosphorylation and whether altered levels of phosphorylation modulate the function or membrane trafficking of this key transporter.

To further address the role of phosphorylation in regulating KCC2 we have assessed whether this protein is directly phosphorylated and whether this covalent modification alters transporter functional expression. Our studies demonstrated that KCC2 is directly phosphorylated by PKC activity on Ser940 within the major C-terminal intracellular domain of this protein. PKC-dependent phosphorylation of Ser940 increased KCC2 cell surface stability and activity by decreasing endocy-
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tosis from the plasma membrane. Endogenous KCC2 expressed in hippocampal neurons was also phosphorylated by PKC activity and, in common with our recombinant studies activation of PKC, increased the accumulation of KCC2 on the neuronal plasma membrane. Together our studies suggest a critical role for PKC-mediated phosphorylation of Ser\(^{840}\) in KCC2 in regulating the functional expression of this transporter in the brain.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal mouse anti-KCC2 antibody clone N1/12 was purchased from the University of California Davis/NIH/NIHM NeuroMaB facility. Polyclonal rabbit anti-KCC2 antibody was purchased from Upstate.

**Biotinylation and Endocytosis Assays**—Cells were washed twice with 1× PBS containing 0.5 mM MgCl\(_2\) and 1 mM CaCl\(_2\) (PBS-CM) and then incubated with 2 ml of 1× PBS-CM containing 1 mg/ml sulfo-NHS-SS-biotin for 30 min for biotin labeling. After labeling, the biotin reaction was quenched by washing three times with 1× PBS-CM containing 50 mM glycine and 0.1% bovine serum albumin (14, 15). Cells were then lysed in 10 mM Na\(_2\)PO\(_4\), pH 7.4, 1% Triton X-100, 0.5% deoxycholate, 10 mM sodium pyrophosphate, 25 mM NaF, 1 mM sodium orthovanadate, 100 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin, leupeptin, and pepstatin, 5 mM EDTA, EGTA, and 100 mM NaCl. Insoluble proteins were removed by centrifugation at 13,200 rpm for 10 min. 50 μg of protein was loaded into a 6% acrylamide gel and resolved by electrophoresis by 160 V for 1 h. The resolved proteins were then electrotransferred at 50 mA onto nitrocellulose membrane over 16 h. The protein blots were blocked by 5% skim milk for 1 h. KCC2 protein was recognized by monoclonal anti-KCC2 antibody (from University of California Davis) at 1 μg/ml concentration diluted in 5% skim milk. Horseradish peroxidase-conjugated donkey anti-mouse secondary antibody was used to recognize the anti-KCC2 antibody at a concentration of 0.2 μg/ml. Chemiluminescence was generated by a VisiGlo™ horseradish peroxidase plus substrate kit from Amresco and detected by an LAS-3000 image reader from Fujifilm. Quantification of the chemiluminescence signal was carried out by Multi Gauge version 3.0 from Fujifilm.

**Immunofluorescence**—HEK-293 cells transfected with KCC2 plasmids or 4-week-old hippocampal neuron cultures were grown on a 1-cm-diameter glass cover slips coated with 1 mg/ml poly-L-lysine. Cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with 1× PBS, and blocked with 1× PBS containing 5% skim milk and 0.2% Triton X-100 for 10 min. The cells were incubated with monoclonal anti-KCC2 antibody for 2 h, washed five times with 1× PBS, incubated with TRITC-conjugated polyclonal anti-mouse antibody in blocking buffer for 1 h, washed five times with 1× PBS, and mounted on glass slides with 3 μl of Vectashield® mounting medium. The prepared slides were imaged with a confocal microscope after 24 h. Acquisition of confocal images was carried out using Laser Sharp 2000 software from Bio-Rad. Quantification of fluorescence images was carried out using MetaMorph software from Universal Imaging Corp.

**In Vitro Kinase Assay**—0.5 μg of fusion protein was incubated with 10 μCi of [\(^{32}\)P]ATP and 1–50 ng of purified PKC or CAMP-dependent protein kinase (PKA) from Calbiochem in a buffer containing 20 mM HEPES (pH 7.5), 10 mM MgCl\(_2\), and 50 μM ATP for 10 min at 30 °C and terminated by the addition of 2× SDS-PAGE sample buffer (18, 19). The reaction mixture was then analyzed by SDS-PAGE. A phosphorimaging device was used to quantify the incorporation of \(^{32}\)P into the proteins.

**Neuronal Cultures**—In brief, rat embryos at embryonic day 18 were removed and decapitated into 1× Hank’s balanced salt solution (HBSS; from Invitrogen) on ice. Brain tissues from the embryos were removed and transferred into fresh HBSS on ice, and hippocampal regions were further dissected out. Dissected hippocampi were placed in 0.25% trypsin at 37 °C for 15 min with gentle shaking. Hippocampi were washed with HBSS two times for 5 min and passed through Pasteur pipettes 10 times to dissociate. Nondissociated debris was allowed to settle to the bottom for 10 min. Dissociated hippocampal neurons were counted by hemocytometer and plated on a 60-mm culture dish using electroporation with a total of 10 μg of DNA and utilized 24–48 h after transfection (17).
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**RESULTS**

In Vitro Analysis of KCC2 Phosphorylation by PKC—Molecular studies have demonstrated that the consensus site for phosphorylation by a number of classical second messenger-dependent protein kinases, including both PKC and PKA, are evident within both the major intracellular domains of KCC2 (3). To commence our studies we expressed the major N-terminal (amino acids 1–102; His-N-KCC2) and C-terminal (amino acids 645–1116; His-C-KCC2) intracellular cytoplasmic domains of KCC2 as His-tagged fusion proteins in *E. coli* (Fig. 1A). Purified fusion proteins were then subjected to *in vitro* kinase assays. This revealed that the His-C-KCC2 fusion protein was phosphorylated by purified PKC to a final stoichiometry of ~0.6 mol/mol, whereas His-N-KCC2 was not phosphorylated under similar conditions (Fig. 1B). In contrast, neither of the fusion proteins was phosphorylated by purified PKA, but the cytoplasmic tail of the GABA<sub>R2</sub> subunit, a previously identified substrate of this enzyme (20), was previously identified under the same conditions (Fig. 1B). Peptide mapping and...
phosphoamino acid analysis revealed that His-C-KCC2 was primarily phosphorylated on serine residues within two major phosphopeptides labeled A and B, respectively (Fig. 1, C and D).

To further analyze KCC2 phosphorylation, site-directed mutagenesis was utilized to convert candidate serine residues within His-C-KCC2 to alanines. Based on the consensus for PKC phosphorylation of (R/K)(X(S/T))X(3-4)(S/T) (where X is any amino acid (21, 22), mutant fusion proteins were produced in which Ser728, Ser940, and Ser1034 were individually and sequentially mutated to alanines. The phosphorylation of the resulting purified proteins by PKC was then compared with that seen for His-C-KCC2. Although mutation of Ser728 and Ser1034 did not significantly alter phosphorylation, mutation of Ser940 reduced phosphorylation to 32.5 ± 2.5% of control. Moreover mutation of Ser940 in combination with either Ser728 or Ser1034 had very similar effects on PKC-dependent phosphorylation compared with mutation of Ser940 alone (Fig. 1E).

The phosphorylation of the 32P-His-C940A/KCC2 was further analyzed using peptide mapping and phosphoamino acid analysis. Significantly, this revealed that mutation of Ser940 ablated peptide A seen on PKC-dependent phosphorylation of His-C-KCC2 (Fig. 1D) and that the remaining sites of phosphorylation for this kinase in His-C940A/KCC2 are serine residues (Fig. 1C). Together these results suggest that the major site for PKC phosphorylation within His-C-KCC2 is Ser940.

**Ser940 Is a Major Site for PKC-dependent Phosphorylation of KCC2 when Expressed in HEK-293 Cells**—To analyze the relevance of our in vitro observations, we transiently expressed KCC2 in HEK-293 cells. KCC2 expression was first analyzed by immunoprecipitation with anti-KCC2 antibody after metabolic labeling with [35S]methionine. This resulted in the isolation of two bands with approximate molecular masses of 125 and 130 kDa from cells expressing wild-type KCC2 but not from cells expressing empty vector. Similar bands were also immunoprecipitated from cells expressing a mutant form of KCC2 in which Ser940 (KCC2940A) was mutated to an alanine (Fig. 2A). The phosphorylation of KCC2 was examined by immunoprecipitation after metabolic labeling with [32P]orthophosphoric acid. A major band of 130 kDa was evident from cells expressing wild-type KCC2 that was not seen in those expressing vector alone, demonstrating basal phosphorylation of this protein in HEK-293 cells. Activation of PKC with phorbol dibutyrate (PDBu) for 10 min produced a significant increase (p < 0.01) in KCC2 phosphorylation of 195.7 ± 5.6% of that evident under basal conditions (Fig. 2B). Phosphorylation of KCC2940A was analyzed using similar methodology, and this mutant construct exhibited robust levels of basal phosphorylation. However in contrast to wild-type KCC2, PDBu did not significantly enhance the phosphorylation of KCC2940A (Fig. 2B). These results are consistent with our in vitro experiments and strongly suggest that the major site for PKC phosphorylation within the C-terminal intracellular domain of this transporter is Ser940.

**Phosphorylation of Ser940 Enhances KCC2 Cell Surface Expression Levels**—To address the functional consequences of KCC2 phosphorylation, we first compared the proportion of KCC2 and KCC2940A expressed on the cell surface of HEK-293 cells using biotinylation (14, 15). This revealed that 22.9 ± 4.5% of wild-type KCC2 was present on the cell surface at steady state, and this could be significantly increased to 40.6 ± 5.4% upon activation of PKC over a 10-min time period, but the total levels of KCC2 expression were not altered under the same conditions (Fig. 2C). In contrast to this 38.7 ± 4.6% of KCC2940A was present on the plasma membrane, a level significantly higher (p < 0.01) than that of wild-type KCC2 (22.9 ± 4.5%; Fig. 2C). However, PDBu treatment did not significantly increase the cell surface expression level of KCC2940A (Fig. 2C).

To confirm the results of our biotinylation assays, we assessed the effects of activating PKC on cell surface expression levels of KCC2 using immunohistochemistry. To do so cells expressing KCC2 or KCC2940A were treated with PDBu, permethylated, and stained with anti-KCC2 antibodies. Confocal images were then recorded from these cells, and the pixel intensity across the entire cell was measured in the presence and absence of PDBU treatment (Fig. 3A). The relative level of membrane expression was determined by calculating the ratio of fluorescence signal associated with the cell periphery and the cytoplasm. Notably treatment of cells expressing wild-type KCC2 with PDBU significantly increased the ratio fluorescence associated with the cell periphery by 182 ± 7.2% of control untreated cells (Fig. 3, B and C). Similar experiments were performed on cells expressing KCC3940A; however, in these cells PDBu did not significantly alter the distribution of KCC2 immunoreactivity (Fig. 3, A–C). Together these biochemical and imaging experiments indicate that phosphorylation of KCC2 on Ser940 increases transporter cell surface expression levels.
PKC-dependent Phosphorylation of Ser940 Decreases KCC2 Endocytosis—To further evaluate the mechanism underlying PKC-dependent modulation of KCC2 cell surface stability, the possible role of this enzyme in regulating transporter endocytosis was evaluated. To do so transfected HEK-293 cells were labeled with NHS-SS-biotin and incubated at 37 °C for up to 20 min in the presence of leupeptin to prevent lysozomal degradation of any internalized protein (14). After cleavage of remaining cell surface NHS-SS-biotin with reduced glutathione, cells were lysed, and internalized biotinylated proteins were isolated on avidin and immunoblotted with KCC2 antibodies. After controlling for the efficiency of cleavage (remaining biotin at 0 min) this approach revealed that the entire cell surface population of KCC2 was endocytosed within 10 min under basal conditions. This process was linear over the initial 5-min period of the assay (Fig. 4A). Under control conditions 80.7 ± 8.2% of the total cell surface population of KCC2 was internalized within 5 min, whereas in the presence of PDBu internalization was significantly reduced (*p < 0.01) to 30.5 ± 4.5% (Fig. 4B).

We used the KCC2S940A mutant to assess the role of Ser940 in mediating the effects of PKC on KCC2 endocytosis. Compared with wild-type KCC2 the endocytosis of this mutant appeared to be significantly decreased over a time course of 20 min in the presence or absence of PKC activation (Fig. 4A). To quantify these results, we compared the level of endocytosis of KCC2 and KCC2S940A after a 5-min incubation at 37 °C in the pres-
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PKC Activity Modulates the Phosphorylation and Cell Surface Stability of Endogenous KCC2 in Cultured Neurons—To examine the relevance of our recombinant studies, we examined the phosphorylation of KCC2 in hippocampal neurons. To initiate these experiments, we first examined the expression of KCC2 in these cells via immunoblotting. A major band of ~130 kDa was seen with anti-KCC2 antibodies that was first detected at 2 weeks and reached maximal expression levels between 4–5 weeks in culture (Fig. 6A) consistent with other studies on the developmental expression of KCC2 in culture (4, 23–25). To examine phosphorylation, 4–5-week cultures were labeled with [32P]orthophosphate for 4 h before treatment with 100 nM PDBu and/or 100 nM Cal for 10 min. Cell lysates were immunoprecipitated (IP) with either control (Con) IgG or KCC2 antibody, and the precipitated material was subjected to SDS-PAGE and visualized using a phosphorimaging device. KCC2 phosphorylation was compared with the level seen under basal conditions (−PDBu), which was assigned a value of 100%. 32P-labeled KCC2 immunopurified from neurons was subjected to phosphoamino acid analysis followed by autoradiography. The migration of phosphoserine (PS), -threonine (PT), and -tyrosine (PY) standards is indicated. D, neurons were treated with or without 100 nM PDBu or 100 nM PDBu + 100 nM Cal for 10 min as indicated and subjected to biotinylation. Cell surface (CS) and Total (T) fractions were then immunoblotted with anti-KCC2 antibody as illustrated in the upper panel. The level of cell surface expression was then compared with that evident under basal conditions (100%), *, significantly different from control (p < 0.01; Student’s t test, n = 3).

The Activity of KCC2 Is Increased by PKC Activation in Cultured Cells—To measure the functional effects of PKC activity on KCC2 function, expressing cells were treated with phorbol-12-myristate-13-acetate (PMA) produced a large and highly significant increase (p < 0.01) in KCC2 activity as measured by furosemide-sensitive [36Rb] influx (Fig. 5). Influx was significantly enhanced (p < 0.01) by a 15-min preincubation with N-ethylmaleimide (NEM), an accepted activator of KCC2 (2) (Fig. 5). Pretreatment of cells with phorbol 12-myristate-13-acetate (PMA) produced a large and highly significant increase (p < 0.01) in KCC2 activity as measured by furosemide-sensitive [36Rb] influx (Fig. 5). To test the role that direct phosphorylation of Ser940 plays in this modulation, the effects of PMA on a number of KCC2 mutants were analyzed. Significantly, the ability of PKC to modulate the activity of KCC2 was blocked by mutation of Ser940, but this treatment did not alter the ability of NEM to stimulate transporter activity (Fig. 5). In contrast mutation of Ser278 or Ser1034 in KCC2 was without effect on either PKC or NEM-dependent stimulation of KCC2 activity, but interestingly mutation of Ser728 appeared to increase constitutive activity of KCC2 (Fig. 5). Together this series of experiments revealed that activation of KCC2 by PKC was dependent upon Ser940, the major site of phosphorylation for this kinase within this protein.

PKC Activity Modulates the Phosphorylation and Cell Surface Stability of Endogenous KCC2 in Cultured Neurons—To examine the relevance of our recombinant studies, we examined the phosphorylation of KCC2 in hippocampal neurons. To initiate these experiments, we first examined the expression of KCC2S940A (Fig. 4B). However, in contrast to KCC2, PDBu treatment did not significantly alter the endocytosis of KCC2S940A (Fig. 4B). These results strongly suggest that the phosphorylation of Ser940 acts to modulate cell surface stability of KCC2 by slowing its endocytosis.

The Activity of KCC2 Is Increased by PKC Activation in Cultured Neurons—To measure the functional effects of PKC activity on KCC2 function, expressing cells were treated with phorbol-12-myristate-13-acetate and N-ethylmaleimide to inhibit endogenous Na+/K+-2Cl– cotransporter and Na+/K+-ATPase, respectively. KCC2 activity was measured by basal furosemide-sensitive [36Rb] influx. In cells expressing KCC2, significant levels of [36Rb] accumulation were evident over a 3–5 min time course that were not seen with cells expressing empty vector (Fig. 5). Influx was significantly enhanced (p < 0.01) by a 15-min preincubation with N-ethylmaleimide (NEM), an accepted activator of KCC2 (2) (Fig. 5). Pretreatment of cells with phorbol 12-myristate-13-acetate (PMA) produced a large and highly significant increase (p < 0.01) in KCC2 activity as measured by furosemide-sensitive [36Rb] influx (Fig. 5). To test the role that direct phosphorylation of Ser940 plays in this modulation, the effects of PMA on a number of KCC2 mutants were analyzed. Significantly, the ability of PKC to modulate the activity of KCC2 was blocked by mutation of Ser940, but this treatment did not alter the ability of NEM to stimulate transporter activity (Fig. 5). In contrast mutation of Ser728 or Ser1034 in KCC2 was without effect on either PKC or NEM-dependent stimulation of KCC2 activity, but interestingly mutation of Ser278 appeared to increase constitutive activity of KCC2 (Fig. 5). Together this series of experiments revealed that activation of KCC2 by PKC was dependent upon Ser940, the major site of phosphorylation for this kinase within this protein.
on mapping PKC-phosphorylation $^{32}$P-His-C-KCC2 together with a neutral peptide (C in Fig. 1D). Together these results suggest that Ser$^{940}$ is likely to represent a site of PKC phosphorylation in neuronal KCC2.

We also examined the effects of PKC activation on the cell surface stability of KCC2 using biotinylation. This revealed that activation of PKC with PDBu produced a highly significant increase ($p < 0.01$) in the proportion of KCC2 expressed on the neuronal cell surface (CS) to 295.6 ± 9.8% of control, an effect that was blocked by a specific PKC inhibitor (Fig. 6D). To confirm our biotinylation experiments, we measured the effects of PKC activation on the targeting of KCC2 to the plasma membrane using immunohistochemistry followed by confocal microscopy (Fig. 7A). The relative level of membrane expression was determined by measuring the ratio of fluorescence signals associated with the cell surface and the cytoplasm of individual proximal dendrites (Fig. 7B). Treatment of neurons with PDBu significantly increased ($p < 0.01$) the level of KCC2 immunoreactivity at the periphery of dendrites compared with control untreated neurons, an effect that was decreased via inhibition of PKC (Fig. 7C). Therefore in common with our recombinant studies these observations demonstrated that KCC2 in its native environment is subjected to PKC-dependent phosphorylation and that this covalent modification increases the targeting of KCC2 to the neuronal cell surface.

**DISCUSSION**

The potassium chloride cotransporter KCC2 is the major determinant of Cl$^-$ transmembrane gradients in adult neurons. The activity of KCC2 results in low intracellular Cl$^-$ concentrations that are responsible for hyperpolarizing responses of GABA$_A$ and Gly receptors in fast synaptic inhibition within the central nervous system (4, 8). Deficits in KCC2 activity are of importance in epilepsy and other central nervous system pathologies (26); therefore comprehending the cellular mechanisms neurons use to regulate the activity of this protein is of significance.

Here we have begun to examine the molecular sites of phosphorylation for individual protein kinases within KCC2 and the role that these residues play in regulating its functional expression. There are a number of studies that have analyzed the regulation of KCC2 by agents that modify the activity of protein kinases and phosphatases (6, 20–27); however, it remains to be demonstrated that KCC2 is actually phosphorylated in neurons. We commenced our analysis by expressing the major N-terminal and C-terminal intracellular domains (residues 645–1116) of KCC2 as fusion proteins in E. coli and analyzed their phosphorylation *in vitro*. This revealed that the C-terminal but not the N-terminal fusion protein was selectively and stoichiometrically phosphorylated by PKC. Peptide mapping and phosphoamino acid analysis revealed that this phosphorylation occurred on serine residues within two major phosphopeptides. Site-specific mutagenesis of Ser$^{940}$ with the C-terminal fusion reduced PKC phosphorylation to 25% of control and ablated one of the major peptides seen on phosphorylation of the wild-type protein. Consistent with our *in vitro* analysis, full-length KCC2 exhibited high levels of basal phosphorylation when expressed in HEK-293 cells as measured via immunoprecipitation after metabolic labeling with $^{32}$P orthophosphoric acid. Moreover, phosphorylation of KCC2 was robustly increased upon activation of PKC, an effect that was ablated by mutation of Ser$^{940}$. Therefore our combined *in vitro* and *in vivo* analysis demonstrated that KCC2 is a substrate of PKC and that the major site of phosphorylation within the major intracellular domain of this transporter is Ser$^{940}$.

In addition to our recombinant experiments, we directly analyzed the phosphorylation of KCC2 in hippocampal neurons using immunoprecipitation. This revealed that under basal conditions in 28–35 days *in vitro* hippocampal neurons, KCC2 was basally phosphorylated on serine and threonine residues. Activation of PKC produced a dramatic increase in the stoichiometry of its phosphorylation to 400% of that seen under basal conditions. Enhanced phosphorylation upon the activation of PKC occurred on serine, threonine, and tyrosine residues. Consistent with our result in hippocampal neurons, we found that KCC2 is phosphorylated on serine/threonine residues in cortical neurons (not shown). In addition, oxidative stress and/or high metabolic activity have recently been reported to induce phosphorylation of KCC2 on tyrosine residues (27). To directly compare our recombinant and neuronal studies of KCC2 phosphorylation, we used peptide mapping. This revealed that KCC2 in hippocampal neurons was phosphorylated within three major tryptic peptides after activation of PKC. Significantly, two of these peptides were also evident on tryptic peptide mapping of the C terminus of KCC2 after PKC phosphorylation *in vitro*. These results together with our recombinant studies suggest that Ser$^{940}$ is phosphorylated in neuronal KCC2 molecules upon the activation of PKC.

The effects of PKC activity on KCC2 functional expression were evaluated using both biochemical and functional

**FIGURE 7.** The subcellular distribution of KCC2 in hippocampal neurons is modulated by PKC activity. A, 4-week-old cultured hippocampal neurons were treated with (+) or without (−) 100 nM PDBu and/or 10 mM Cal for 10 min. Neurons were fixed, permeabilized, and stained with anti-KCC2 antibody coupled to a rhodamine-conjugated secondary. Confocal images were then acquired from the bottom to the top of proximal dendrites. In the enlargements (insets) the region used for quantification are indicated by the lines, and the numbers 1 and 2 represent the points at which data acquisition commenced and terminated, respectively. B, the pixel intensity per unit length (0.1 μm) was then calculated for cells treated with PDBu (gray line) and PDBu/Cal (broken black line) and for control cells (solid black line). C, the ratio of plasma membrane to cytosol signals was calculated, and values were then compared with levels seen under control conditions (−PDBu; 100%). ***, significantly different from control ($p < 0.01$; Student’s t test, $n = 10$ cells from three differing transfections).
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approaches. As measured by biotinylation, activation of PKC increased the cell surface expression levels of KCC2 by ~200% relative to control over a 10-min time period. This effect of PKC on KCC2 steady state cell surface expression levels was critically dependent on Ser940, as mutation of this residue abrogated the effect of activating PKC upon transporter cell surface stability. Likewise immunohistochemistry revealed that activation of PKC increased the targeting of PKC to the periphery of HEK-293 cells, an effect that was also dependent upon Ser940. To analyze the mechanism underlying the effects of Ser940 phosphorylation on cell surface stability, the effects of PKC activity on KCC2 transporter endocytosis were measured. Under basal conditions the entire cell surface population of KCC2 was internalized over a time course of 10 min. Activation of PKC dramatically slowed the endocytosis of KCC2 compared with control, an effect that was critically dependent on Ser940, consistent with the higher steady state levels expression of this mutant. Previous experiments using Xenopus oocytes have illustrated that the activation of PKC decreases cell surface expression levels and activity of KCC2 (6). The varying effects of PMA may result from differing trafficking itineraries between oocytes and HEK-293 cells or the varying incubation temperatures of 18 and 37 °C, respectively. However, and in keeping with our results, the effects of PMA in oocytes was dependent on Ser940, suggesting a critical role for this residue in regulating KCC2 functional expression via PKC activity. As Ser940 is not conserved in other CCCs (3), these results suggest a unique role for this residue in regulating KCC2 function. The effects of PKC activity on the cell surface stability of KCC2 on the neuronal plasma membrane were examined. Using both biotinylation and imaging, it was evident that activation of PKC in hippocampal neurons that had been cultured for 4–5 weeks produced a large and highly significant increase in the cell surface expression levels of KCC2 within 10 min. Whether these effects were mediated via altered endocytosis remains to be ascertained; however, it is interesting to note that studies in hippocampal neurons have previously shown that under basal conditions 50% of cell surface KCC2 is degraded within 20 min. Enhancing PKC-dependent phosphorylation may thus provide a rapid and dynamic mechanism to enhance the cell surface activity of KCC2. In keeping with this potential mechanism, Fiurnelli et al. (28) have demonstrated that EC1 shifts caused by changing [Ca2+]c, are dependent on PKC activity. However if these effects are mediated by changes in the stoichiometry of KCC2, phosphorylation has not been demonstrated.

Our studies have shown that KCC2 is phosphorylated directly by PKC on Ser940 within the cytoplasmic C-terminal domain of this critical transporter and that phosphorylation of this residue acts to increase KCC2 functional expression by slowing its endocytosis. Therefore, cell signaling molecules that activate PKC signaling pathways may have profound effects on neuronal Cl− homeostasis by regulating the phosphorylation and functional expression of KCC2. Given the critical role KCC2 plays in regulating Cl− homeostasis, this phospho-dependent functional modulation may have significant consequences for the efficacy of synaptic inhibition mediated by GABA_A and Gly receptors.

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