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Chemical Crosslinking Studies with the Mouse Kcc1 K-Cl Cotransporter

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Abstract

Oligomerization, function, and regulation of unmodified mouse Kcc1 K-Cl cotransporter was studied by chemical crosslinking. Treatment of Xenopus oocytes and 293T cells expressing K-Cl cotransporter Kcc1 with several types of chemical cross-linkers shifted Kcc1 polypeptide to higher molecular weight forms. More extensive studies were performed with the amine-reactive disuccinyl suberate (DSS) and with the sulfhydryl-reactive bis-maleimidohexane (BMH). Kcc1 cross-linking was time-dependent in intact oocytes, and was independent of protein concentration in detergent lysates from oocytes or 293T cells. Kcc1 crosslinking by the cleavable cross-linker DTME was reversible. The N-terminal and C-terminal cytoplasmic tails of Kcc1 were not essential for Kcc1 crosslinking. PFO-PAGE and gel filtration revealed oligomeric states of uncrosslinked KCC1 corresponding in mobility to that of cross-linked protein. DSS and BMH each inhibited KCC1-mediated $^{86}Rb^{+}$ uptake stimulated by hypotonicity or by N-ethylmaleimide (NEM) without reduction in nominal surface abundance of KCC1. These data add to evidence supporting the oligomeric state of KCC polypeptides.

Keywords

Potassium chloride cotransport; disuccinimidyl suberate; bismaleimidohexane; Xenopus oocyte; pentadecafluorooctanoic acid PAGE

Introduction

The Slc12/Ccc cation chloride cotransporter gene superfamily includes at least nine members in mammalian species, with homologs are expressed in yeast, bacteria, and archaea. Mammalian Slc12 genes include four Kcc genes (Kcc1–4) encoding Na^{+}-independent K-Cl

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cotransporters, in addition to the Na-K-Cl cotransporters Nkcc1 and Nkcc2, and the Na-Cl cotransporter, Ncc [1]. K-Cl cotransporters regulate neuronal and glial electrochemical equilibrium potential for Cl-, and so can determine the excitatory or inhibitory influences of GABA- and glycine-gated Cl^- channels [2]. The $Kcc2^{-/-}$ mouse dies perinatally due to respiratory failure consequent to loss of synaptic inhibitory input [3]. Human KCC3 mutations are associated with syndromic agenesis of the corpus callosum, and the mouse knockout phenocopies the human disease [4]. The $Kcc4^{-/-}$ mouse exhibits deafness and renal tubular acidosis [5].

The Kcc and Nkcc transporters play opposing roles in the acute and chronic regulation of cell volume through volume decrease and increase, respectively [2;6]. Kcc activating stimuli include hypotonic and isotonic swelling, sulfhydryl alkylation by N-ethylmaleimide (NEM), serine-threonine kinase inhibitors such as staurosporine, hypertonic urea (but not hypertonic NaCl), and acid pH. Kcc inhibitors include serine-threonine phosphatase inhibitors and loop diuretics [7]. These various regulatory stimuli are mediated in part by the kinases Osr1, Spak1, Wnk3, and Wnk4 [2;8]. Elevated K-Cl cotransport activity in red cells genetically altered in cytoskeletal proteins [9] also suggests a tonic inhibitory role for cytoskeletal anchoring.

The important role of K-Cl cotransport in erythrocyte volume regulation is magnified in hemoglobinopathies characterized by red cell dehydration, such as HbS and HbC disease and β-thalassemia. Therapeutic inhibition of K-Cl cotransport has been studied as an adjunct treatment for these disorders [10;11]. Kcc3 and Kcc1 appear to be the predominant K-Cl cotransporter polypeptides of erythrocytes. Whereas Kcc3 mediates nearly all mouse erythroid K-Cl cotransport activity [12], KCC3 deficiency in human red cells selectively decreases cotransport stimulated by NEM, while leaving staurosporine-stimulated transport unaltered [13].

Much remains to be learned about Kcc polypeptide structure. Chemical cross-linking data has suggested a homodimeric structure for NKCC1 [14], but a hetero-oligomeric structure was supported by the abilities of SLC12A9/CIP to coimmunoprecipitate NKCC1 and to suppress its function [15]. A hetero-oligomeric structure was also proposed to explain cAMP-sensitive dominant-negative functional inhibition of the NKCC2 variant mBSC1-A9 by mBSC1-A4 [16]. Both homo- and hetero-oligomeric structures were suggested by the ability of a dominant negative form of Kcc1 (Δ_N117) to co-immunopreciptate wildtype Kcc1, and to inhibit function of coexpressed Kcc2, KCC3, and Kcc4 [17]. More recently, epitope-tagged rabbit Kcc1 was shown to undergo homo- and hetero-oligomeric interactions with other epitope-tagged Kcc isoforms as well as with Nkcc1 [18]. However, none of the cation chloride cotransporters have yet displayed a sigmoidal concentration vs. activity relationships that would support a kinetically important intersubunit interaction. Moreover, the possible regulation of KCC oligomerization state during acute regulation of ion transport rate remains unknown.

We hypothesized that chemical cross-linking might provide insight into the effect of Kcc1 oligomerization state on transporter function and regulation. We therefore have characterized the effect of chemical cross-linkers on recombinant, untagged, mouse Kcc1 polypeptides. The results provide independent evidence for a native oligomeric structure of mouse Kcc1 and other Kcc polypeptides.

Materials and Methods

cRNA expression in Xenopus oocytes

Oocytes were harvested from female Xenopus laevis, and defolliculated as previously described [17;19]. On the same or next day, oocytes were microinjected with 50 nl water or cRNA (12.5 ng in water) transcribed with the T7 Megascript kit (Ambion, Austin, TX) from

linearized cDNA templates encoding mouse Kcc1 [17;19], rat Kcc2, human Kcc3a, and mouse Kcc4 [17]. Oocytes were then incubated at 19° for 2–3 days in ND-96 containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 Na pyruvate, and gentamicin (5 mg%), titrated to a final pH of 7.40. Incubation medium was replaced daily.

Chemical cross-linking in intact Xenopus oocytes

Groups of 3–5 intact oocytes previously injected with cRNA or water were incubated 40 min at 20° in ND-96 lacking pyruvate and gentamicin (cross-linking buffer) and containing the following crosslinkers (from Pierce, Rockford, IL) at the noted concentrations (unless otherwise indicated): 5 mM bis(sulfosuccinimidyl) suberate (BS^3), or 2 mM disuccinyl suberate (DSS), dithio-bis-maleimidoethane (DTME), disuccinimidyl tartarate (DST), ethylene glycolbis (succinimidylsuccinate) (EGS), bis-maleimidohexane (BMH), succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB), or 2,2'-di-isothiocyanatostilbene-4,4'-disulfonate (DIDS, Calbiochem). The water-insoluble cross-linkers DSS, DST, BMH, DTME, EGS, and SMPB were made up in DMSO (final concentration 0.4%). The water-soluble cross-linkers BS^3 and DIDS were made up fresh at their final concentrations in ND96.

In some experiments, groups of 5–10 oocytes were preincubated at 20° in isotonic ND-96, in hypotonic medium (ND-72), in hypertonic medium (ND-200), or in ND-96 containing either 1 mM NEM or 200 μM (R(+)-[2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-indenyl-5-yl-)oxy]acetic acid (DIOA, Sigma). Some oocytes underwent 40 min additional incubation in the same solutions containing 2 mM DSS or BMH.

After rinsing, oocytes were homogenized in immunoprecipitation buffer (IP buffer, 10 μl/oocyte) of 1% Triton X-100 containing (in mM) 250 NaCl, 50 Tris-HCl, pH 8.0, 1 EDTA, 1 phenylmethylsulfonylfluoride (PMSF), and Boehringer protease inhibitor tablets per manufacturer's recommendation. These detergent lysates were incubated with shaking for 30 min at 4°, then centrifuged 10 min in a microfuge. The resulting supernatants were subjected to SDS-PAGE, and the fractionated proteins were transferred to nitrocellulose membrane. KCC1 was detected by immunoblot as previously described, using affinity-purified rabbit polyclonal antibodies to the mouse KCC1 acetylated N-terminal aa 1–14 and to KCC1 C-terminal aa 1074–1086 [17;19]. Immunoblots were developed by enhanced chemiluminescence (Perkin-Elmer/NEN, Boston, MA).

Reversible cross-linking of metabolically labeled Kcc1

Oocytes were co-injected with ^{35}S-methionine (1 mCi/ml) and either water or Kcc1 cRNA. 2–3 days later, groups of 10 labeled oocytes were incubated for 40 min at 37° in ND-96 containing DTME (2 mM), then washed and homogenized in IP buffer. Lysate supernatants prepared as above were diluted with an equal volume of NaCl-free IP buffer, precleared with 5% normal rabbit serum, then incubated overnight with 1:10 diluted affinity-purified rabbit polyclonal antibody raised against the acetylated N-terminal peptide of mouse Kcc1. Immune complexes precipitated with Protein A-agarose were washed 6 times in IP buffer, 6 more times in NaCl-free IP buffer without NaCl, subjected to SDS-PAGE in the absence or presence of 5% β-mercaptoethanol, then to ^{35}S-Met fluorography.

Cross-linking in lysates from oocytes and from 293T cells

Groups of 3–8 oocytes were solubilized in 1% Triton X-100 immunoprecipitation buffer (10 μl/oocyte) containing (in mM) 250 NaCl, 20 Na phosphate, 1 EDTA, 1 PMSF. The resulting detergent lysate was incubated with shaking at 4° for 30 min, then centrifuged for 10 min in a microfuge. The lysate supernatant was incubated with 2 mM cross-linker for 3 hrs at 4°. Amine-reactive cross-linkers were then quenched with 20 mM Tris-Cl, and the samples were subjected to SDS-PAGE and immunoblot analysis.

HEK-293T cells were transfected with mouse Kcc1 cDNA in pcDNA3, using Lipofectamine 2000 (Invitrogen). Cells were subjected to G418 selection (200 μg/ml) in DMEM plus 10% calf serum containing 2 mM glutamine, 100 μM penicillin and 100 μM streptomycin. Resistant populations were isolated with cloning rings, expanded, and screened by immunoblot for Kcc1 expression. A cell line overexpressing Kcc1 was subjected to detergent extraction, and the resulting lysate supernatant was prepared and incubated with cross-linker as described above. Lysates were subjected to SDS-PAGE and immunoblot analysis.

Gel filtration of oocyte lysates

A Superose 6HR 10/30 gel filtration column (Pharmacia) equilibrated with 0.2% reduced Triton X-100 buffer of pH 7.40 containing (in mM) 20 Na phosphate 150 NaCl, and 1 EDTA was calibrated with globular proteins of the BioRad Gel Filtration Standards: thyroglobulin (670 kDa), immunoglobulin G (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and with cyanocobalamin (1355 Da). Oocyte detergent lysate supernatants were subjected to gel filtration at a flow rate of 0.2 ml/min using a Pharmacia FPLC system. Fractions of 0.5 ml were collected, and 7 μl samples of each fraction were analyzed by SDS-PAGE and immunoblot.

PFO-PAGE

Ten oocytes were homogenized in 100 μl buffer containing (in mM) 250 NaCl, 50 Tris-HCl, 1 EDTA, 1 PMSF, and 0.25–1.0% pentadecafluorooctanoic acid (PFO, Oakwood Products, W. Columbia, SC). To some samples, SDS was added after homogenization as indicated (Fig. 5). PFO-PAGE [20]was performed at 4°C with 0–8% PFO in the 2x sample buffer, and 0.5% PFO in the running buffer, or as indicated with electrophoresis at 140 V. Crosslinked phosphorylase *b* and High-molecular-weight Rainbow markers (Sigma) served as M_r standards. Fractionated proteins were subjected to immunoblot analysis.

Kcc1 immunofluorescence detection in Xenopus oocytes

Confocal immunofluorescence microscopy was performed as described previously [17]. Five days after cRNA injection, 6–10 oocytes were subjected to 1 hr incubation in ND-96 in the absence or presence of DSS or BMH, then were fixed in 140 mM NaCl, 10 mM Na phosphate, pH 7.4 (PBS) containing 3% paraformaldehyde at 20° for 4 hrs. After extensive rinsing in PBS, oocytes were pre-treated with 1% SDS for 5 min, they were blocked with 1% BSA, 0.05% saponin in PBS for 1 hr, then incubated overnight with affinity purified anti-Kcc1 N-terminal antibody. Oocytes were washed, incubated overnight with Cy3-coupled anti-Ig secondary antibody (Jackson Immunoresearch, Westport, PA), then again extensively washed. After dehydration in methanol for 1 hr, followed by overnight incubation in BA:BB oocyte clarification solution [17], the oocytes were subjected to confocal imaging with the BioRad 1024.

Kcc1-mediated ^{86}Rb influx

Kcc1 function was measured in Xenopus oocytes as unidirectional ^{86}Rb as previously described [17]. Oocytes were cross-linked at 20° as described above, in the absence or presence of 2 mM DSS, 2 mM BMH, then washed and quenched. These oocytes were then incubated 30 min in the presence of isotonic or hypotonic medium (ND-72), prior to addition of ^{86}Rb to initiate the 1 hr influx period.

Results

Cross-linking of Kcc1 in Xenopus oocytes by amine-reactive cross-linkers

Intact oocytes expressing functional mouse Kcc1 at the cell surface (17,21) were exposed individually to several cross-linkers. Since Kcc1 exofacial loops are predicted to contain lysine

residues, the amino-reactive, homo-bifunctional, water-soluble cross-linkers BS^3 (5 mM) and DIDS (2 mM) were tested, but produced no evident cross-linking of Kcc1 (not shown). The amino-reactive, homo-bifunctional, water-insoluble, cell-permeant cross-linkers DSS, DST, and EGS were also tested. As shown in Fig. 1A, DSS treatment of intact oocytes generated a new Kcc1-immunoreactive band of ~ 200 kDa, approximately twice the M_r of the 1085 aa Kcc1 monomer. Formation of this band was time-dependent (Fig. 1B; in some experiments cross-linking was evident within 5 min, not shown).

Exposure to DSS of Triton X-100 extract prepared from Kcc1-expressing Xenopus oocytes generated a cross-linked band of the same M_r as that generated in intact oocytes (Fig. 1C). The oligomeric state of Kcc1 was stable in Triton X-100, since the relative extent of cross-linking by DSS was not attenuated by 10-fold dilution of input protein. In some experiments, appearance of the cross-linked Kcc1 band was accompanied by decreased intensity of the monomeric Kcc1 immunoblot band.

Cross-linking of Kcc1 in Xenopus oocytes by sulfhydryl-reactive cross-linkers

Mouse Kcc1 expressed in intact Xenopus oocytes was also cross-linked by the water-insoluble, cell-permeant, homobifunctional sulfhydryl cross-linker BMH. BMH produced a major cross-linked Kcc1 band of M_r ~ 200 kDa, lower than that produced by DSS (Fig. 2A). Combined treatment of oocytes with DSS + BMH yielded both KCC1 cross-link products, but did not produce a cross-linked KCC1 band of yet higher M_r. However, cross-linking with BMH or with DSS of Triton X-100 lysate from Kcc1-expressing oocytes yielded in each case predominant cross-linked Kcc1 products with similar M_r values of ~ 200 kDa (Fig. 2B). Combined treatment of oocyte lysate with DSS + BMH yielded, in addition to this M_r ~ 200 kDa band a Kcc1 band of yet higher M_r (Fig. 2B), and higher than that produced by exposure of intact oocytes to the combined cross-linkers (Fig. 2A). The cross-linked Kcc1 product produced by the amine-reactive cross-linker EGS was of the same M_r as that produced by DSS treatment (Fig. 2C). In contrast the heterobifunctional cross-linker SMPB produced cross-linked Kcc1 bands corresponding to both the DSS and BMH bands, as well as a slightly more prominent band of intermediate M_r. DST, only 6.4 Å in length, was an ineffective Kcc1 cross-linker (Fig. 2C). As the more effective cross-linkers tested ranged in length between 11.4 and 16.1, this result may reflect the intermonomeric distance between target amines and target sulfhydryls on adjacent Kcc1 monomers, or between Kcc1 and another binding protein.

The prolonged exposure of Kcc1 to Triton X-100 accompanying the immunoprecipitation protocol led to SDS-resistant oligomerization secondary to intrinsic disulfide bond formation, as indicated by its sensitivity to electrophoresis in the presence of β-mercaptoethanol (Fig. 2D). The reversible, homobifunctional, sulfhydryl cross-linker DTME cross-linked Kcc1 to a product of M_r much higher than that of the un-crosslinked apparent KCC1 dimer which predominated in non-reducing conditions (Fig. 2D, rightmost lane). This covalent cross-linking was completely reversed by the presence of β-mercaptoethanol during SDS-PAGE.

Kcc1 cross-linking was evident not only in Xenopus oocytes. When cross-linkers were incubated with Triton X-100 lysates from 293T cells overexpressing mouse Kcc1, BMH and DSS produced cross-linked products of the same M_r, whose abundance was not attenuated by 10-fold dilution of protein concentration (Fig. 2E). BS^3 produced the same Kcc1 cross-linked product at even higher efficiency (Fig. 2E), although BS^3 was inactive as a Kcc1 cross-linker in intact oocytes, suggesting either a cytoplasmic target site on Kcc1, or a site rendered accessible in Triton X-100.

Susceptibility to cross-linking of other Kcc polypeptides

Intact oocytes expressing mouse Kcc4, human KCC3a, and rat Kcc2, were also subjected to cross-linking. DSS effectively cross-linked Kcc4 and Kcc2 to a single band of M_r ~250 kDa, but less effectively cross-linked KCC3a (Fig. 3A, 3B). BMH weakly cross-linked KCC4, but failed to cross-link KCC3 (Fig. 3A). Thus, Kcc4, KCC3a, and Kcc2 likely share similar homo-oligomeric states as judged by their susceptibility to cross-linking.

To determine the contributions of the cytoplasmic domains of Kcc1 to cross-linking susceptibility, intact oocytes expressing either Δ_N117 Kcc1 or Δ_C660 Kcc1 were exposed to cross-linkers (Fig. 3B). The former lacks the N-terminalc cytoplasmic tail of Kcc1, and the latter lacks the Kcc1 C-terminal cytoplasmic tail. Both mutants are expressed at or near the oocyte cell surface [17]. The N-terminal cytoplasmic tail was not required for cross-linking by either BMH or by DSS, but the M_r difference between the BMH and DSS products was less pronounced than for those of wild-type Kcc1. Although absence of the C-terminal cytoplasmic tail did not prevent cross-linking by the sulfhydryl reactive BMH, the cross-linking yield of amine-reactive DSS was substantially reduced. The difference in M_r between BMH and DSS cross-linking products of wildtype Kcc1 was not preserved in the Δ_C660 Kcc1 oligomers. The M_r values of cross-linked products of truncated Kcc1 polypeptides were consistent with those of homodimers.

PFO PAGE of Kcc1

Fig. 4 compares the M_r of Kcc1 in SDS with that in the less denaturing detergent, PFO, used previously to study native oligomeric structure of ion channels and transporters [20;[21];22] and non-membrane proteins [22]. The M_r of monomeric Kcc1 solubilized from Xenopus oocytes in all tested concentrations of PFO resembled that of completely monomeric Kcc1 in 4% PFO in the presence of SDS. (three rightmost lanes of Fig. 4. However, all PFO concentrations tested in the absence of SDS featured an additional, prominent Kcc1 band of M_r ~250 kDa, and a fainter band at M_r ~400 kDa. These KCC1 bands were suggestive of dimmers and tetramers. However, Kcc1 from oocytes cross-linked with DSS or BMH did not exhibit a detectable ~400 kDa band.

Gel filtration studies of Kcc1

Kcc1 was solubilized from oocytes in 1% Triton X-100 and fractionated on a Superose 6HR 10/30 column in 0.2% Triton X-100. Kcc1 (calculated monomeric molecular weight of 120623 Da) exhibited a broad elution profile with a leading edge in fraction 10, connected by a broad shoulder to the major peak in fractions 13 and 14, and tailing into fractions 15 and 16, Fig. 5A). The elution profile of Kcc1 similarly solubilized from oocytes after exposure to the cross-linker DSS exhibited a shift to larger apparent molecular mass. Fractions 10–12 analyzed by SDS-PAGE exhibited covalently cross-linked bands of a Kcc1 complex of M_r ~500 kDa (Fig. 5B, as calibrated by soluble, globular proteins). This covalently cross-linked Kcc1 eluted in association with monomeric Kcc1.

Effects of chemical cross-linking on Kcc1 surface expression and function

Fig. 6A shows that expression of Kcc1 at or near the oocyte surface was not reduced by the 40 min cross-linking protocol used in these studies, whether with the amine-reactive DSS or the sulfhydryl-reactive BMH. In contrast, treatment of intact oocytes with cross-linkers inhibited stimulation of Kcc1-mediated K-Cl cotransport (Fig. 6B). DSS pretreatment inhibited 92% of hypotonicity-stimulated K-Cl cotransport, and 73% of that stimulated by NEM. BMH pretreatment inhibited 83% of hypotonicity-stimulated K-Cl cotransport, and 69% of that stimulated by NEM.

Effects of functional stimulation and inhibition upon Kcc1 cross-linking

Functional inhibition of Kcc1 by cross-linking prompted consideration of altered Kcc1 oligomeric state as a possible regulator of Kcc1 activity. We therefore tested the effects of stimulators and inhibitors of K-Cl cotransport activity on the chemical cross-linking of Kcc1. Fig. 7A shows that hypotonic stimulation decreased the extent of Kcc1 cross-linking by DSS but not by BMH. NEM stimulation of oocytes had no effect on Kcc1 cross-linking by DSS, but increased the M_r of the BMH-crosslinked Kcc1 band (leftmost lane). The inhibitory stimulus of hypertonicity did not alter cross-linking by either DSS or BMH.

Ligand binding or modulating conditions can stabilize either active or inactive cross-linked conformations of ion channels [23] and transporters [24]. Fig. 7B shows that exposure to the transport inhibitor DIOA, whether in isotonic (basal) conditions or in the stimulating condition of hypotonicity, increased abundance of the Kcc1 cross-linking product of BMH. A lesser increase is evident for the DSS-cross-linked KCC1 product.

Discussion

Our results show that unmodified mouse Kcc1 expressed in Xenopus oocytes or in 293T cell exists in an oligomeric structure. This structure is demonstrated by time-dependent chemical cross-linking of Kcc1 to higher M_r forms in intact Xenopus oocytes. The oligomeric state is maintained after solubilization of Kcc1 in 1% Triton X100. Cross-linking of KCC1 after solubilization in this detergent was independent of protein concentration, consistent with a native oligomeric state. Cross-linking in detergent was also independent of heterologous expression system of origin, since it also occurred with Kcc1 overexpressed in 293T cells. Kcc4 and Kcc2 were also susceptible to cross-linking, as was KCC3a to a lesser extent. These results were consistent with our earlier demonstration that Δ_N117 Kcc1 could serve as a dominant negative functional inhibitor of Kcc1, Kcc2, Kcc3, and Kcc4 [17].

Cytoplasmic N- and C-terminal tails of Kcc1 are not needed for cross-linking of Kcc1

Both Δ_N117 Kcc1 and Δ_C660 Kcc1 polypeptides retained susceptibility to cross-linking by BMH and DSS (fig. 3B), suggesting that the transmembrane domain harbored target site amines and Cys residues for these cross-linkers. However, DSS reactivity was diminished in Δ_C660 Kcc1 compared to wildtype Kcc1. The diminished difference in M_r between BMH- and DSS-cross-linked products of the truncated proteins compared to those of wildtype Kcc1 suggests either a different preferential target sites or distinct SDS-resistant conformations dependent on the presence of one or other of the cytoplasmic domains. The results are consistent with the lack of homomeric interaction of the isolated C-terminal cytoplasmic tails of rabbit Kcc1 and of human KCC3a in a yeast two-hybrid system [18]. Kcc N-terminal tails could not be evaluated in that system.

Particularities of Kcc1 cross-linkers and cross-linking environments

When cross-linking in intact oocytes was evaluated by autoradiography of metabolically labeled protein in immunoprecipitates, monomer depletion reliably accompanied cross-linking (Fig. 2D). Similarly, when Kcc1 was cross-linked in Triton X-100 extract, monomer generally decreased in proportion to the amount of oligomer generated (Fig. 2B, 2E). However, this was less consistently the case for KCC1 cross-linked in intact oocytes (i.e. Fig. 1B, Fig. 3). We observed similarly variable increases in total immunoreactive protein in our study of chemical crosslinking of the Ae2 Cl^-/HCO_3^- exchanger [25]. In-membrane cross-linking may produce alter the native oligomeric conformation of Kcc1 so as to increase subsequent antibody accessibility of the N-terminal epitope, even in the presence of SDS.

The amine cross-linker DSS and the sulfhydryl cross-linker BMH produced Kcc1 complexes of indistinguishable M_r when Kcc1 was cross-linked in 1% Triton extracts, but of distinct size when cross-linked in intact oocytes. The hetero-bifunctional cross-linker SMPB cross-linked Kcc1 to an M_r intermediate to those of DSS and BMH. Whereas Kcc1 cross-linking in intact oocytes and in Triton extract was of equivalent efficacy for BMH, efficacy was higher in Triton extract for DSS. Both DSS and BMH inhibited physiological stimulation of Kcc1-mediated K-Cl cotransport.

The distinct M_r values of Kcc1 cross-linked by BMH and by DSS likely reflects distinct structures of the SDS-denatured cross-linked oligomers resulting from distinct reaction sites of the cross-linker. The reactivity of these sites appear to differ in the membrane bilayer in intact oocytes and in detergent solution. Cross-linking in intact oocytes also may reflect distinct Kcc1 conformations in the plasmalemma and in intracellular organelles, as detected for the AE1 Cl^-/HCO_3^- exchanger [26].

Native oligomeric state of unmodified Kcc1

Both gel filtration and PFO-PAGE provided evidence for stable Kcc1 oligomers in the absence of cross-linking. Non-crosslinked Kcc1 eluted with two peaks, consistent with monomer and dimer, or (if the calibration curve based on globular, soluble proteins applies) with dimer and tetramer. DSS-cross-linked Kcc1 eluted exclusively in the higher M_r peak. Cross-linked Kcc1 in fractions 10–12 of Fig. 5B can be interpreted as covalent links either between monomers or dimers. Evidence consistent with a "dimer of dimers" is provided by PFO-PAGE at low PFO concentration. However, Kcc1 cross-linked with DSS or BMH in intact oocytes prior to solubilization was refractory for formation of the high molecular weight band suggestive of a dimer of dimers (Fig. 4). Non-Kcc polypeptides may also contribute to some of the observed cross-linked Kcc products, especially to the very high M_r Kcc1 products of the reversible crosslinker DTME (Fig. 2D). The yield of immunoprecipitated cross-linked Kcc1 as blotted to PVDF was insufficient for mass spectrometric analysis to address this possibility.

Recombinant, epitope-tagged rabbit Kcc1 was similarly shown to be exist in a homo-oligomeric state, and could also be co-precipitated with co-expressed epitope-taggted forms of human KCC3a, rat Kcc2, mouse Kcc4, or human NKCC1 [18].

Is regulation of K-Cl cotransport accompanied by altered KCC oligomeric state?

DSS and BMH each inhibited hypotonic and NEM-mediated stimulation of Kcc1-mediated $^{86}Rb^+$ uptake (Fig. 6B) without decreasing Kcc1 polypeptide expression at or near the oocyte surface (Fig. 6A). Although the non-specific actions of the cross-linkers might well have modified cytoskeletal structure or plasticity, this functional inhibition is consistent with the possibility that K-Cl cotransport activation accompanies (and might be caused by dissociation from higher-order oligomer to lower order oligomer or monomer. Hypotonic stimulation of Kcc1 decreased cross-linking by DSS, although that by BMH was unchanged. NEM stimulation of Kcc1 was associated with an increase in M_r of the BMH-cross-linked Kcc1 band (Fig. 7A), Although NEM is believed to target the inhibitory volume-sensitive kinase(s) [27], it may also covalently modify sulfhydryl residues of Kcc1 [28] and/or alter their accessibility to BMH. Alternatively, NEM pre-treatment may regulate Kcc1 interaction with a distinct binding protein. The Kcc inhibitor, DIOA, enhanced Kcc1 cross-linking (Fig. 7B), perhaps by stabilizing a conformation that approximates DSS target sites on interacting Kcc1 monomers to facilitate cross-linking.

Regulation of ion transport activity through control of transporter oligomeric state has been considered for NHE Na^+/H^+ exchangers. Although chemical cross-linking of NHE3 in renal brush border membrane vesicles was not detectably altered by conditions which activate

transport [29], a change in oligomeric state remains a possible explanation for the slow allosteric activation of NHE3 by acid pH [30]. The more rapid, allosteric activation of NHE1 by acid pH and hyperosmolarity has been modeled as reflecting conformational change of homodimers without alteration of oligomeric state [31].

Further information about the native oligomeric state and supramolecular complexes of Kcc1 and other Kcc polypeptides expressed in erythroid and other cell types will await ongoing proteomic and structural analysis, as informed by rapidly evolving models of kinase and phosphatase regulation of K-Cl cotransporters.

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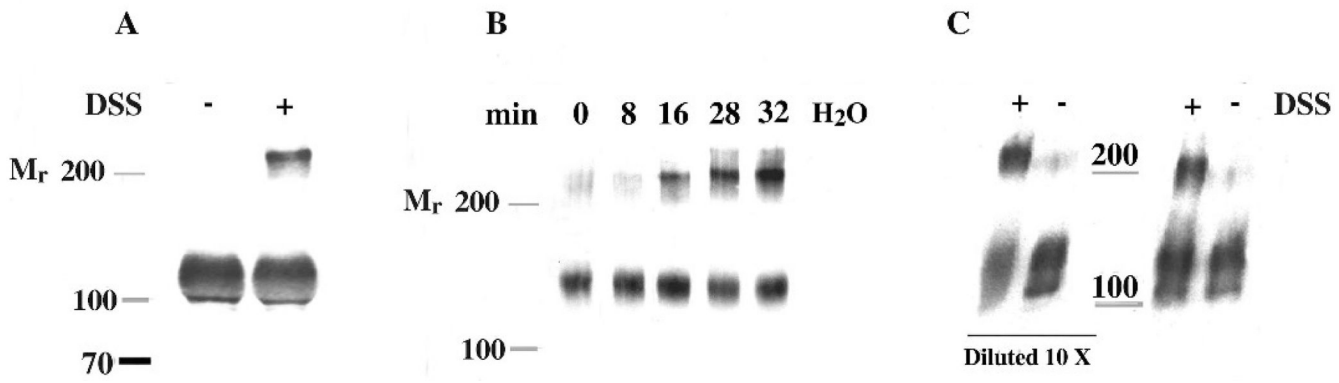


Fig. 1. Initial characterization of DSS cross-linking of Kcc1

A. Kcc1 from intact Xenopus oocytes exposed to 2 mM DSS for 40 min. **B.** DSS Cross-linking of Kcc1 in intact oocytes by DSS (2 mM) was time-dependent. **C.** Cross-linking of Kcc1 by DSS (2 mM) in Triton X-100 oocyte lysate supernatants for 3 hrs at 4°C. Cross-linking was not diminished by 10-fold dilution of lysate. Immunoblots of 5% SDS-PAGE developed with antibody to KCC1 N-terminal peptide. Each lane was loaded with lysate from 0.1 oocyte. Identical results were obtained using antibody to Kcc1 C-terminal peptide (not shown). Representative of at least 4 four similar experiments.

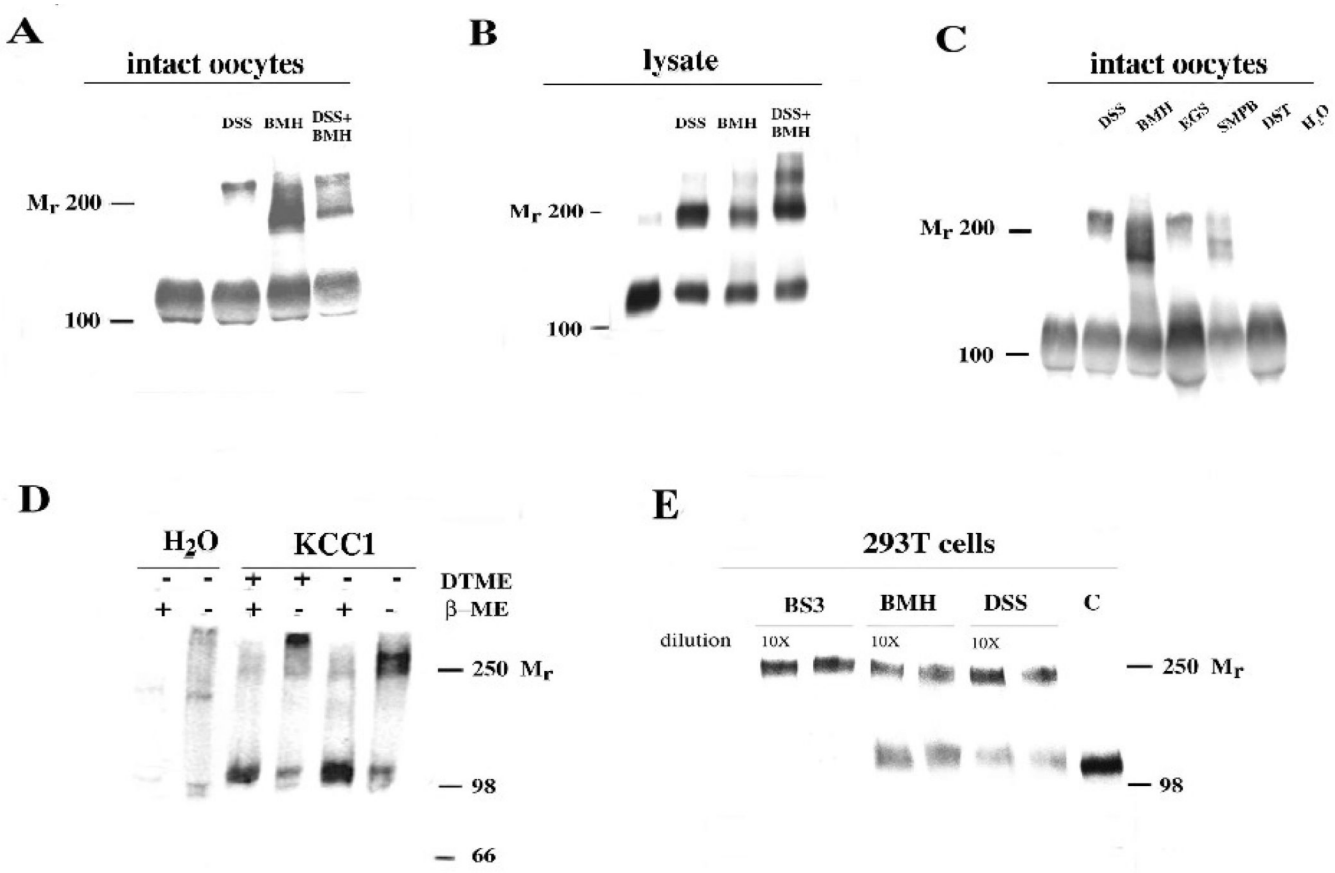


Fig. 2. Kcc1 is cross-linked by multiple cross-linkers

A. Treatment of intact oocytes with DSS or with BMH (2 mM, 40 min, 20°C) produced Kcc1 complexes of different M_r values. **B.** Treatment of Triton X-100 oocyte lysate supernatants with DSS or with BMH (2 mM, 3 hrs, 4°C) produced Kcc1 complexes of similar M_r values, and treatment with both cross-linkers increased the abundance of a higher M_r species. **C.** Treatment of intact oocytes with EGS (2 mM, 40 min, 20°C) produced a Kcc1 complex of M_r similar to that produced by DSS. Intact oocyte treatment with SMPB produced a Kcc1 complex of distinct M_r. DST was ineffective as a KCC1 cross-linker. Each lane in panels A–C was loaded with lysate from 0.1 oocyte. **D.** ^{35}S-methionine-labeled oocytes, previously injected with water or with Kcc1 cRNA, were treated with or without the reversible sulfhydryl cross-linker DTME (2 mM, 40 min, 20°C), then solubilized in Triton X-100. KCC1 immunoprecipitates from oocyte lysate supernatants were resolved by SDS-PAGE in reducing and non-reducing conditions (each lane was loaded with material from 10 oocytes). **E.** Treatment of Triton X-100 lysates from Kcc1-expressing 293T cells with DSS, BMH (2 mM), or BS^3 (5 mM, 4 hrs, 4°C) produced similar Kcc1 complexes of high M_r, whose abundance was not diminished by 10-fold dilution. Immunoblots of 5% SDS-PAGE were developed with antibody to Kcc1 N-terminal peptide. Representative of two (C) or three or more similar experiments (A, B, D, E).

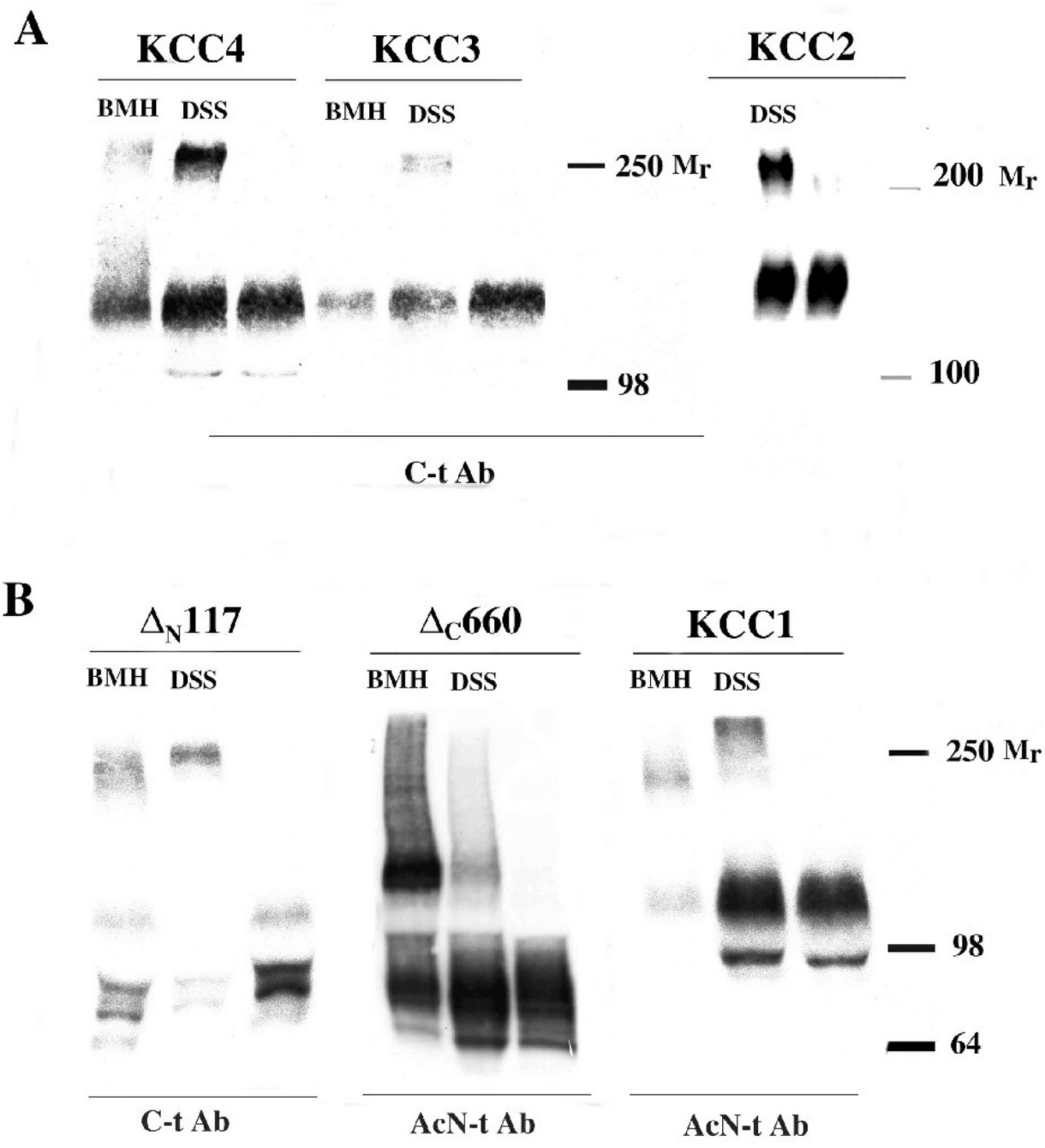


Fig. 3. Other Kcc transporters and truncated forms of Kcc1 can be cross-linked

A. Rat Kcc4 expressed in intact oocytes was cross-linked by DSS, but less effectively by BMH. Human KCC3a was crosslinked less well than Kcc4 by DSS. No cross-linked KCC3a was detected after BMH treatment. Rat Kcc2 was effectively cross-linked by DSS. One of two experiments. **B.** Mouse Kcc1 Δ_N117 (lacking the N-terminal cytoplasmic domain) was efficiently crosslinked by DSS and by BMH. Mouse Kcc1 Δ_C660 (lacking the C-terminal cytoplasmic domain) was more efficiently cross-linked by BMH than by DSS. Immunoblots of 5% SDS-PAGE developed with the indicated anti-Kcc1 antibodies. Each lane was loaded with lysate from 0.1 oocyte. Representative of three similar experiments.

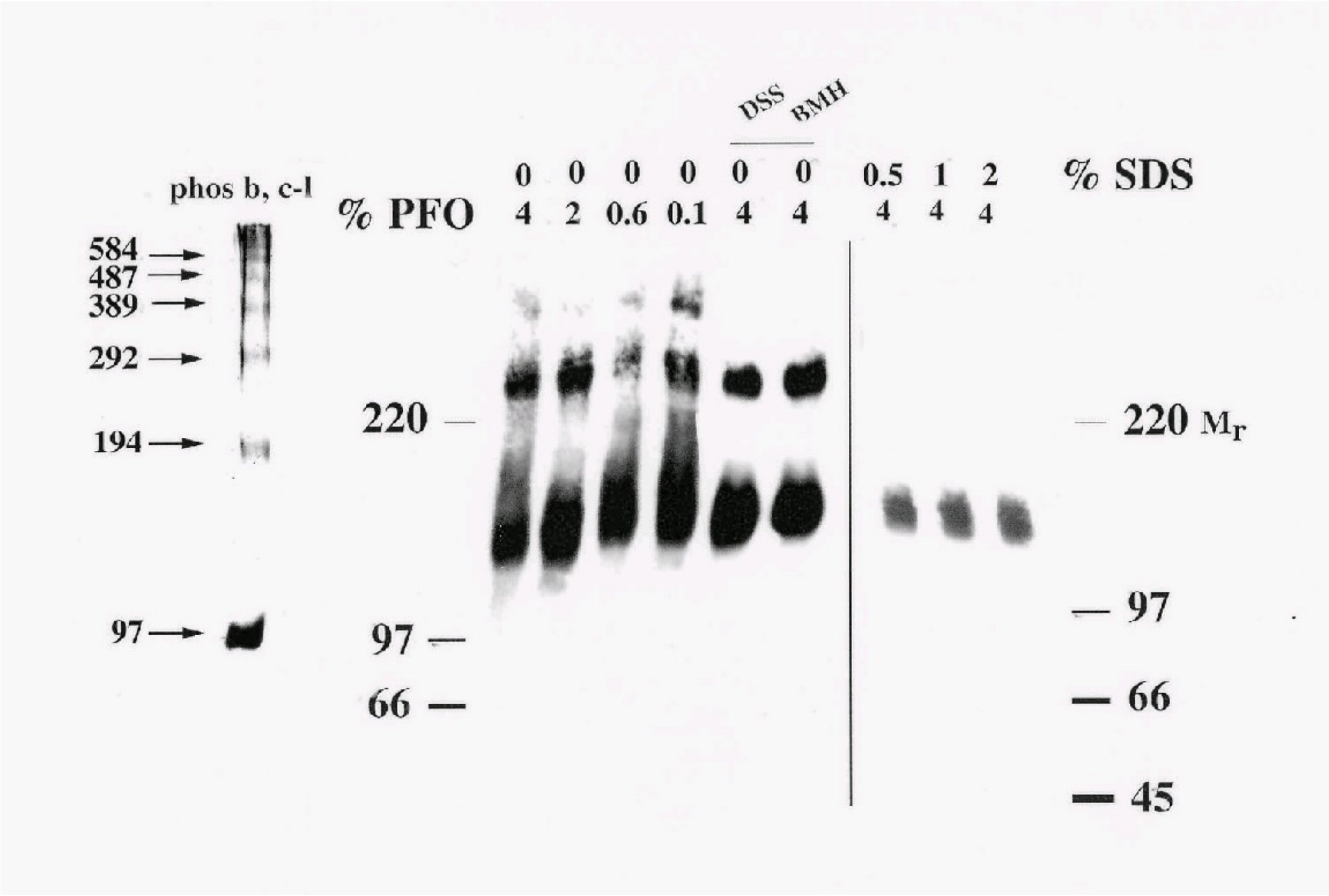


Fig. 4. PFO-PAGE of Kcc1

Oocytes expressing Kcc1 were solubilized for 10 min in the indicated concentrations of PFO without or with (rightmost three lanes) additional SDS. Middle lanes indicate previous cross-linking of intact oocytes with 2 mM DSS or BMH for 40 min at 20°C. Detergent lysate supernatants of were fractionated by 6% PFO-PAGE, and subjected to immunoblot. M_r standards were cross-linked phosphorylase *b* (left lane, extending from 97 to 584 kDa) and Rainbow High Molecular Weight Markers (Sigma), extending from 220 to 45 kDa (right). Each lane was loaded with lysate from 0.1 oocyte. One of three similar experiments.

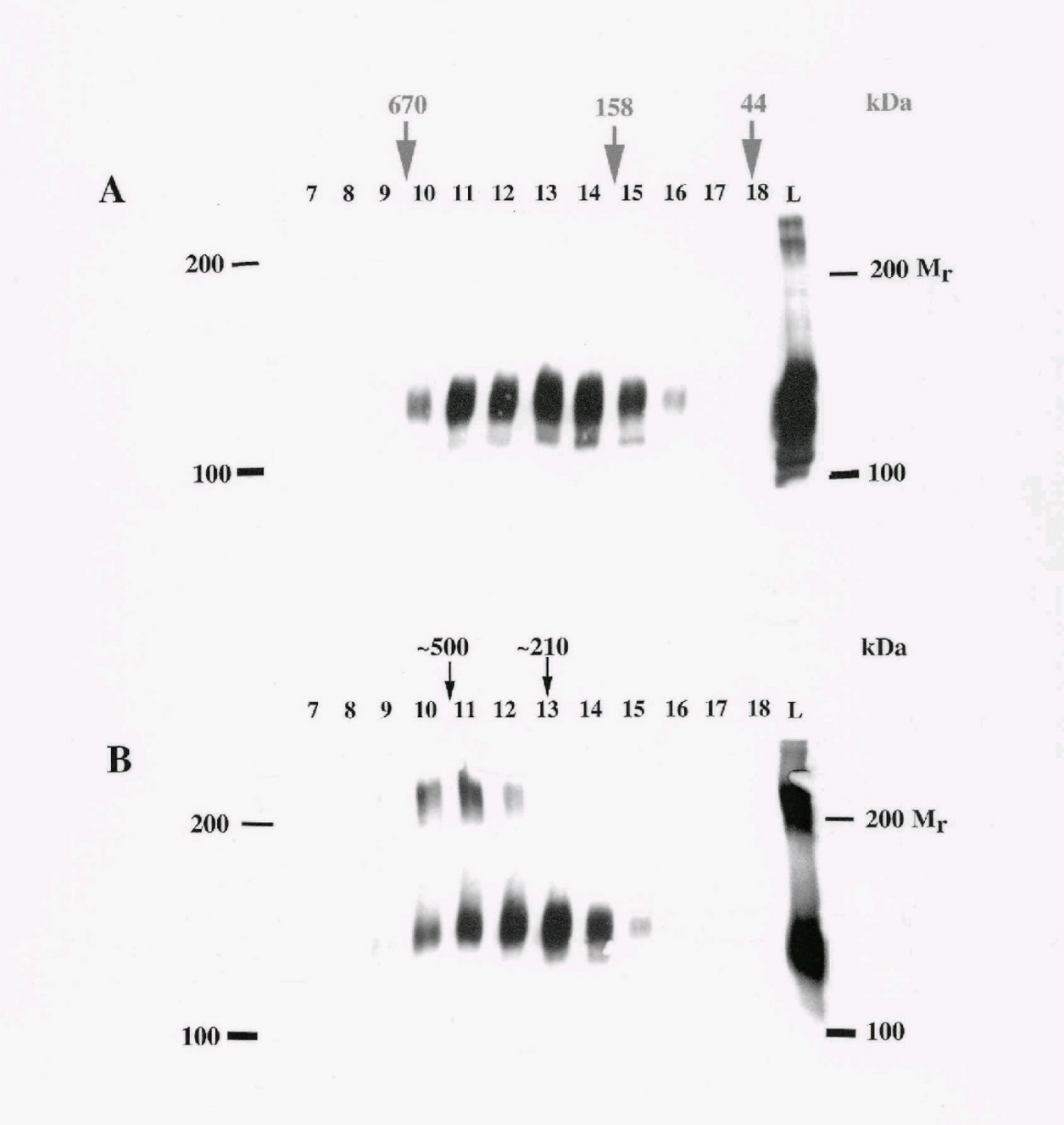


Fig. 5. Gel filtration profiles of Kcc1 in lysates from intact oocytes previously untreated (A) or treated with DSS (B)

Triton lysate from 20 Kcc1-expressing oocytes was loaded on the column. Each lane represents 1.4% of its corresponding fraction volume. Grey arrows (A) indicate peaks of soluble globular M_r marker proteins. Black arrows (B) indicate approximate M_r of peaks of Kcc1 monomer and dimer. Immunoblots are representative of at least three similar elution profiles. Rightmost lanes (L) were loaded with supernatant from Triton X-100 oocyte lysate of 0.7 oocytes.

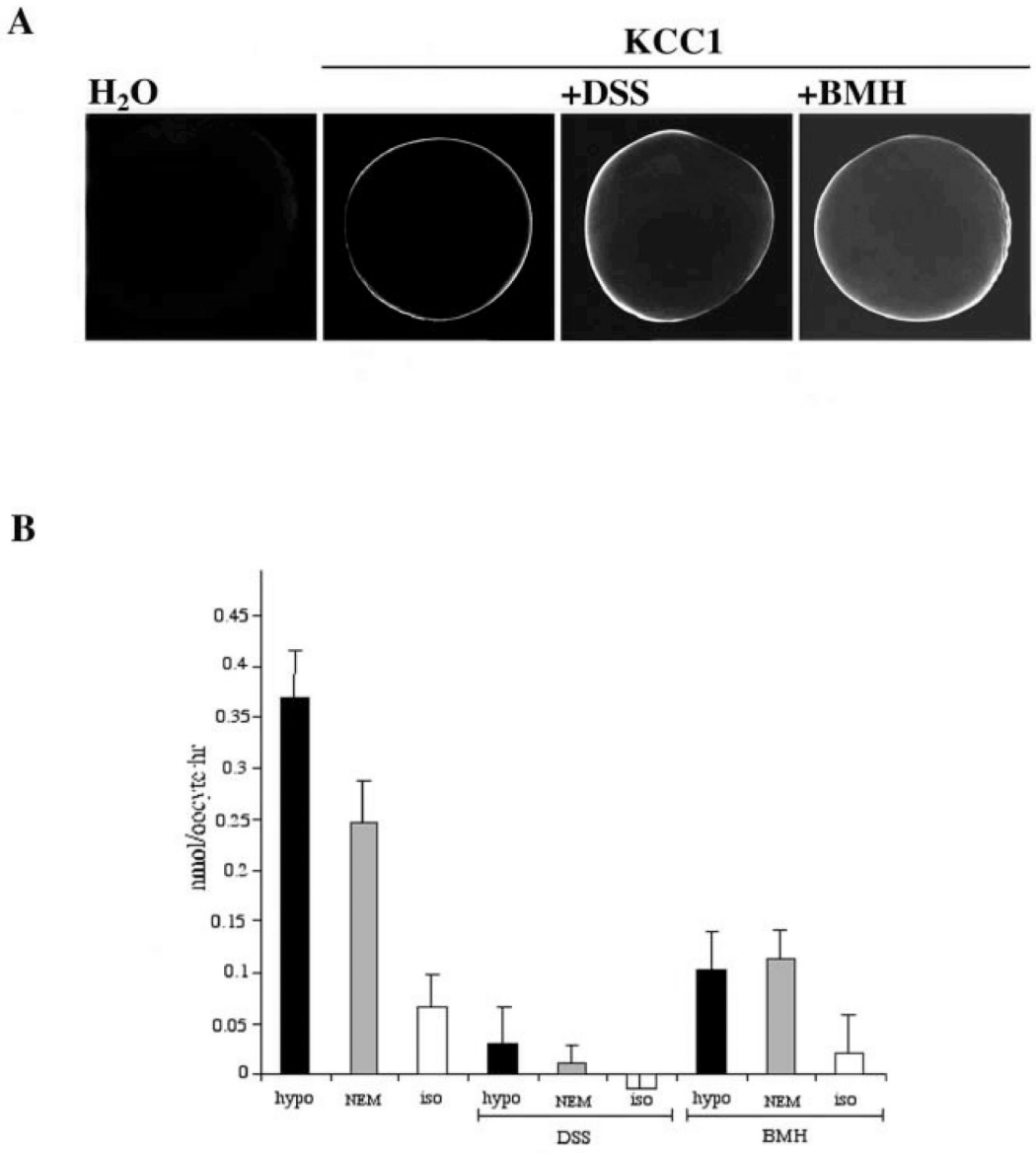
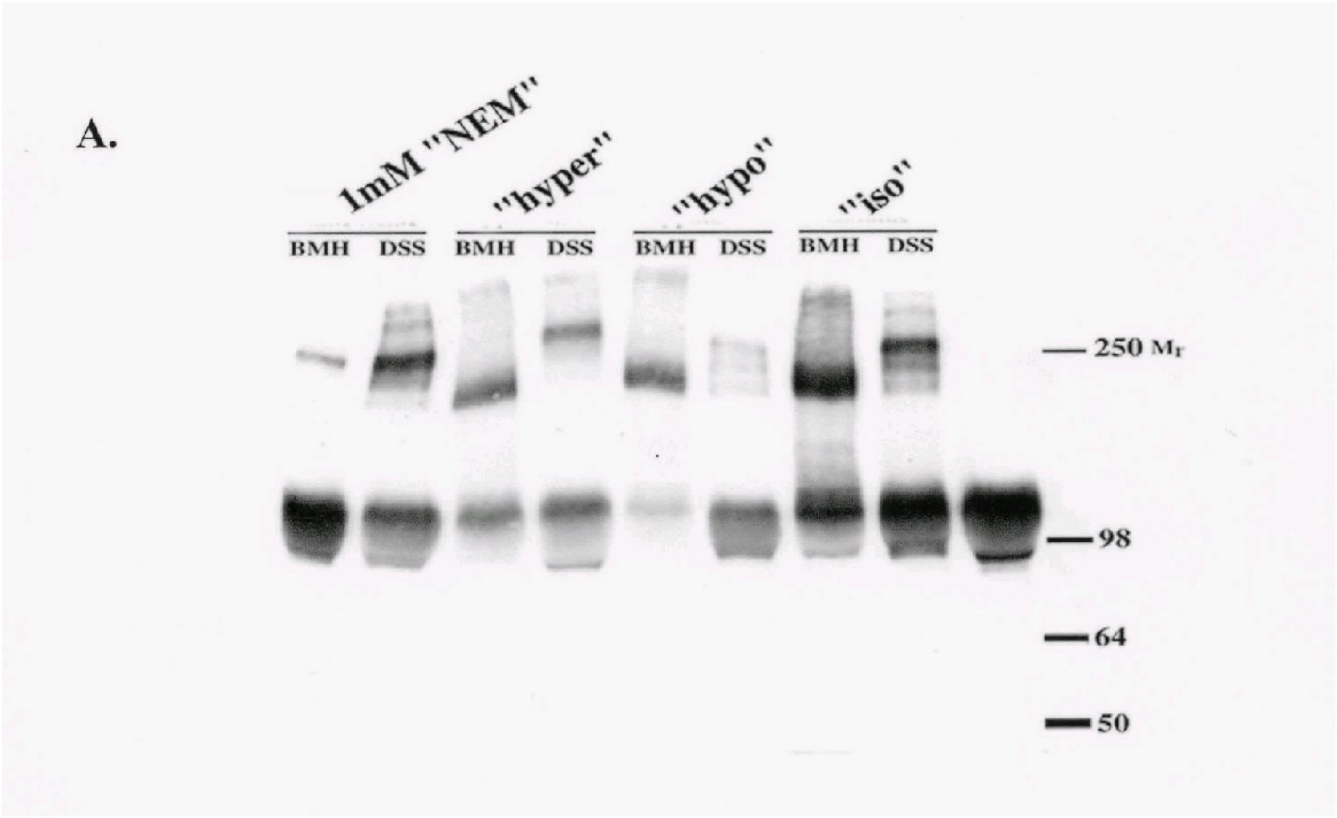


Fig. 6.

A. Effect of cross-linkers on Kcc1 surface expression. Treatment of oocytes with DSS or with BMH (2 mM, 40 min, 20°C) did not decrease abundance of Kcc1 at or near the oocyte surface. Representative of 8 water-injected and 10–12 Kcc1-injected oocytes fixed in each condition. **B. Effect of cross-linkers on Kcc1-mediated K-Cl cotransport activity.** Kcc1-expressing oocytes were first treated without or with DSS or BMH (2 mM, 40 min, 20°C), then stimulated with hypotonicity or NEM as indicated. Influx values were corrected for fluxes measured identically in control oocytes previously injected with water. (n) oocytes from 4 experiments with DSS, and 2 with BMH.



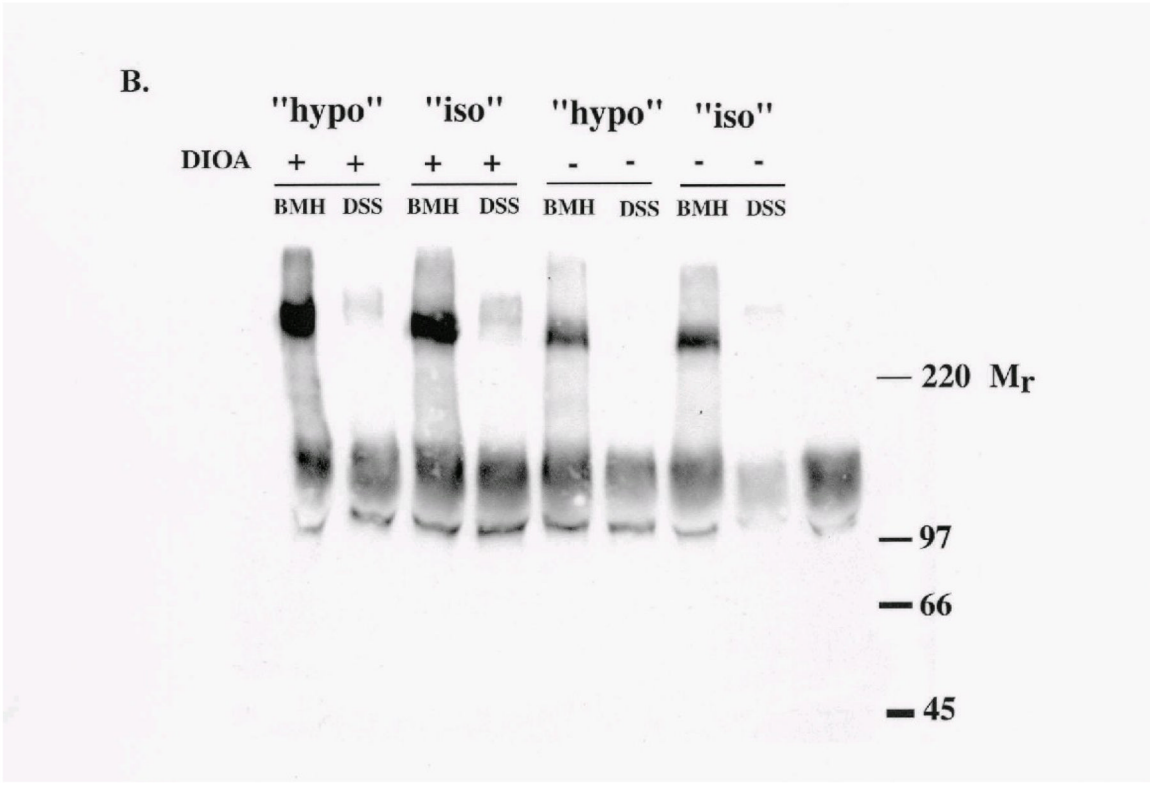


Fig. 7.

A. Effect of K-Cl cotransport activation on Kcc1 cross-linking in intact oocytes. Cross-linking by DSS or by BMH (each at 2 mM for 40 min at 20°C) was affected minimally by prior hypotonic swelling or hypertonic shrinkage respectively to activate or to inhibit K-Cl cotransport. However, cross-linked Kcc1 bands produced by cross-linking with DSS and with BMH were of similar M_r after K-Cl cotransport stimulation with NEM. Each lane was loaded with lysate from 0.1 oocyte. One of three similar experiments. **B. Effect of DIOA on Kcc1 cross-linking.** Each lane was loaded with lysate from 0.1 oocyte. One of two similar experiments.