Assembly of Retinal Rod or Cone Na^+/Ca^{2+}-K^+ Exchanger Oligomers with cGMP-Gated Channel Subunits as Probed with Heterologously Expressed cDNAs†

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ABSTRACT: Proper control of intracellular free Ca^{2+} is thought to involve subsets of proteins that co-localize to mediate coordinated Ca^{2+} entry and Ca^{2+} extrusion. The outer segments of vertebrate rod and cone photoreceptors present one example: Ca^{2+} influx is exclusively mediated via cGMP-gated channels (CNG), whereas the Na^+/Ca^{2+}-K^+ exchanger (NCKX) is the only Ca^{2+} extrusion protein present. In situ, a rod NCKX homodimer and a CNG heterotetramer are thought to be part of a single protein complex. However, NCKX−NCKX and NCKX−CNG interactions have been described so far only in bovine rod outer segment membranes. We have used thiol-specific cross-linking and co-immunoprecipitation to examine NCKX self-assembly and CNG−NCKX co-assembly after heterologous expression of either the rod or cone NCKX/CNG isoforms. Co-immunoprecipitation clearly demonstrated both NCKX homo-oligomerization and interactions between NCKX and CNG. The NCKX−NCKX and NCKX−CNG interactions were observed for both the rod and the cone isoforms. Thiol-specific cross-linking led to rod NCKX1 dimers and to cone NCKX2 adducts of an apparent molecular weight higher than that predicted for a NCKX2 dimer. The mass of the cross-link product critically depended on the location of the particular cysteine residue used by the cross-linker, and we cannot exclude that NCKX forms a higher oligomer. The NCKX−NCKX and NCKX−CNG interactions were not isoform-specific (i.e., rod NCKX could interact with cone NCKX, rod NCKX could interact with cone CNGA, and vice versa). Deletion of the two large hydrophilic loops from the NCKX protein did not abolish the NCKX oligomerization, suggesting that it is mediated by the highly conserved transmembrane spanning segments.

Na^+/Ca^{2+} exchange plays a crucial role in Ca^{2+} homeostasis of most cells (recently reviewed in ref *1*). Vertebrate rod photoreceptor cells express a distinct K^+-dependent Na^+/Ca^{2+}-K^+ exchanger that utilizes the K^+ outward gradient in addition to the Na^+ inward gradient to transport Ca^{2+} out of the cell (NCKX)[1] (*2*, *3*). There is very little homology between the amino acid sequences of the NCKX and the K^+-independent Na^+/Ca^{2+} exchanger (NCX) (*4*). In the past few years, NCKX cDNAs have been cloned from retinal rod and cone photoreceptors from several vertebrate species (*4*−*8*), from nonretinal cells (*9*−*11*), and from lower organisms such as *Drosophila* (*12*), *Caenorhabditis elegans* (*13*), and sea urchin (*14*).

In the outer segments of rod and cone photoreceptors, Ca^{2+} enters the cell exclusively through the cGMP-gated channels (CNG) in the plasma membrane. The CNG channels of cone and rod photoreceptors differ, however, significantly in the cyclic nucleotide affinity, Ca^{2+} permeability, and Ca^{2+} blockage (*15*, *16*). Moreover, Ca^{2+} extrusion occurs much faster in cones than in rods (*17*, *18*). The specific properties of both the CNG channel and NCKX and their spatial localization in the cell bring about the characteristic differences between the two types of photoreceptors. In the outer segments of bovine rod photoreceptors, NCKX1 has been reported to occur as a dimer (*19*), and this dimer has been shown to be part of a larger complex with CNG as well as other proteins (*20*−*22*). Here, we have used chemical cross-linking and co-immunoprecipitation to examine oligomerization of rod and cone NCKX expressed in insect cells, with and without coexpression with the respective CNG channel. Recently, it has been shown that the rod CNG channel is a heteromultimer containing three CNGA1 subunits and one CNGB1 subunit (*23*−*25*). Furthermore, the CNGA1 subunit interacts with NCKX1 in bovine rod photoreceptors (*21*). The CNGA, but not the CNGB subunit by itself, forms a functional cGMP-gated channel, whereas the CNGB subunit imparts several additional properties to the CNG channel

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[1] Abbreviations: NCKX, Na^+/Ca^{2+}-K^+ exchanger; NCKX1, rod Na^+/Ca^{2+}-K^+ exchanger; NCKX2, cone Na^+/Ca^{2+}-K^+ exchanger; CNG, cGMP-gated channel; CNGA, CNG α subunit; CNGB, CNG β subunit; CNGA1, CNGA subunit of rod CNG; CNGA3, CNGA subunit of cone CNG; NCX, Na^+/Ca^{2+} exchanger; pPDM, *N*,*N*′-*p*-phenylene dimaleimide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; MW, molecular weight.

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(reviewed in ref *26*). Nothing is known about a possible interaction of cone NCKX2 and the cone CNG channels. Our study shows that both heterologously expressed rod and cone NCKX readily form larger complexes upon cross-linking using a thiol-specific cross-linker; co-immunoprecipitation shows that both rod and cone NCKX can form homo-oligomers as well as NCKX−CNGA hetero-oligomers. We examine domains important for oligomerization of both proteins, and moreover, we examine the isoform specificity of oligomerization.

MATERIALS AND METHODS

cDNA Clones and Insertion of Epitope Tags. The different rod and cone NCKX and CNGA clones used have been described before: chicken rod NCKX1 (accession number AAF25808) and chicken cone NCKX2 (accession number AAF25810) (*7*); chicken rod CNGA1 (accession number: X89599) and cone CNGA3 (accession number: X89598) (*27*). Using appropriate restriction sites, the human Myc epitope tag was inserted into the N-terminus of the NCKX cDNAs used, and the 6H2 epitope tag was inserted into the N-terminus of the CNGA cDNAs; correct insertion was verified by sequencing of all PCR and oligonucleotide generated fragments. The resulting constructs were cloned into the pEIA vector (*13*). The human Myc tag (EQKLISEEDL) was inserted after residue Thr93 of chicken rod NCKX1 or after residue Pro98 of chicken cone NCKX2, respectively. The 6H2 epitope (part of the N-terminus of bovine NCX1, SHVDHISAETEMEGEGNETGECTGSYYCKKGVILPIWEDEP) was inserted after residue Gln47 of the chicken cone CNGA and after residue Pro27 of chicken rod CNGA, respectively. A number of chicken rod−cone NCKX chimeras and deletion mutants were constructed. For this purpose, a XhoI site was generated at the TM1−cytoplasmic loop junction in both rod and cone NCKX. This site was used to generate rod−cone chimeras and for deletion of the cytosolic loop. A HindIII site was generated at the cytosolic loop−TM2 junction to delete the cytosolic loop. The above 6H2 linker was inserted to provide for a spacer in place of the deleted cytosolic loop. The junctions at the XhoI site are FMKFNVQVLE289 for chicken cone NCKX2 and TMKHNVSLE283; the junctions at the HindIII site are 459LSLAWPD for chicken cone NCKX2 and 461LSLEWPE. The 41 residue 6H2 linker (see above) was dropped in to replace the cytosolic loops of about 180 residues. Plasmid DNA, for transfection of insect cells, was prepared using the EndoFree Plasmid Maxi Kit (Qiagen).

Thiol-Specific Cross-Linking and Western Blotting. High Five insect cells (BTI-TN-5B1-4 cells, High Five; Invitrogen, Carlsbad, CA) were stably transfected with various NCKX and/or CNGA channel cDNA as described (*13*). Transfected cells were cultured in IPL41 medium supplemented with 10% fetal bovine serum (heat inactivated) at 28 °C. The cells were harvested by centrifugation for 5 min at 300*g* in an IEC Centra CL2 centrifuge and washed twice in 50 mL of Na-buffer (150 mM NaCl, 20 mM Hepes/arginine pH 7.4, 80 mM sucrose, 200 μM EDTA). The cells were resuspended in Na-buffer and divided into different test tubes for cross-linking under different conditions, as described in the figure legends. Thiol-specific cross-linking was carried out with up to 50 μM pPDM (*N*,*N*′-*p*-phenylene dimaleimide), either at room temperature or at 4 °C for 5−10 min, and stopped by adding 3 mM DTT (final concentration). In control experiments, cysteine residues were modified with 10 μM NEM under the same conditions. After chemical modification, the cells were sedimented at 300*g* at 4 °C for 10 min. Membranes were solubilized for 20 min on ice in RIPA buffer containing 1% Triton X-100, 0.5% DOC, 140 mM NaCl, 25 mM Tris pH 7.5, 10 mM EDTA, one protease inhibitor tablet/10 mL (Complete, Mini, Roche), and sedimented for 10 min at 20 000*g* at 4 °C. The supernatant was retrieved, and the protein content was determined with the Bradford assay. Gel electrophoresis was performed in a 8.5% Laemmli constant gel system with a 4% stacking gel. The proteins were transferred onto a nitrocellulose membrane and probed with appropriate antibodies.

Immunoprecipitation. To test for possible interactions between NCKX and CNGA, High Five cells or HEK293 cells were transfected transiently with cDNAs encoding the indicated NCKX and/or CNGA cDNAs. Two days after transfection, cells were harvested and washed several times by centrifugation at 300*g* for 4 min. Membranes were solubilized in RIPA buffer. Solubilized membranes were sedimented at 20 000*g*, and the protein concentration in the supernatant was determined with the Bradford assay. Solubilized membranes containing 400−1600 μg of protein were incubated for 2 h at 4 °C with 5 μL of 6H2 monoclonal antibody (0.5 mg/mL, Novus) or 1 μL of polyclonal Myc antibody (1 mg/mL, Cell Signaling). Next, 20 μL of a 25% slurry of protein A/G PLUS-Agarose beads (Santa Cruz Biotech) was added, the volume was adjusted to 500 μL with PBST (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20, pH 7.3), and the mixture was incubated overnight at 4 °C under continuous mixing. The precipitate was collected by centrifugation at 1000*g* for 3 min and washed three times with 1 mL of PBST. Finally, the immunoprecipitate was extracted with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 41 mM DTT, and 0.03% (w/v) phenol red), and proteins were separated on a 8.5% Laemmli gel with a 4% stacking gel.

RESULTS

Role of CNGA in Thiol-Specific Cross-Linking of the Chicken Rod or Cone NCKX in Insect Cells. It has been shown that the CNG channel and the NCKX1 in bovine rod outer segment membranes are associated in a complex, and it is thought that the CNGA1 subunit of the CNG channel interacts with NCKX1 (*21*, *22*). Moreover, thiol-specific reagents were found to efficiently cross-link the NCKX1 to a homo-dimer in the plasma membrane, but very little cross-linking was observed after solubilization of NCKX1 in CHAPS or after reconstitution of NCKX1 into lipid vesicles (*19*). These findings suggest that the interaction between NCKX1 and the CNGA1 subunit could be assessed by the efficiency of NCKX1 dimerization as probed by thiol-specific cross-linking. On the basis of these findings on rod NCKX1 in situ, we were interested in examining whether heterologously expressed rod NCKX1 proteins could be cross-linked to dimers and whether NCKX1 cross-linking was facilitated by coexpression with the corresponding CNGA1 subunit as it had been shown that bovine rod CNGA1 binds to bovine rod NCKX1 (*21*). Nothing is known about the possible interaction between cone NCKX (NCKX2) and the cone

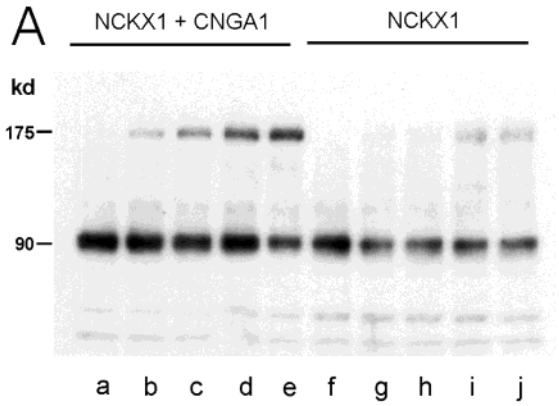

FIGURE 1: Thiol-specific cross-linking of chicken rod NCKX1 (containing a Myc epitope) under stringent conditions. NCKX1 is visualized with an antibody against the Myc epitope. Cells coexpressing NCKX1 and CNGA1 subunit (lanes a−e) or only the NCKX1 (lanes f−j) were cross-linked with increasing concentrations of pPDM for 10 min at 4 °C. Lanes a and f are controls where the cysteines were modified with 10 μM NEM. The pPDM concentrations were 2.5 μM (lanes b and g), 5 μM (lanes c and h), 7.5 μM (lanes d and i), and 10 μM (lanes e and j). The total protein load was 30 μg per lane. Note that the apparent MW of chicken rod NCKX1 in this experiment (~90 kDa) was a bit higher than that observed in most experiments (80−83 kDa; e.g., Figures 6 and 7).

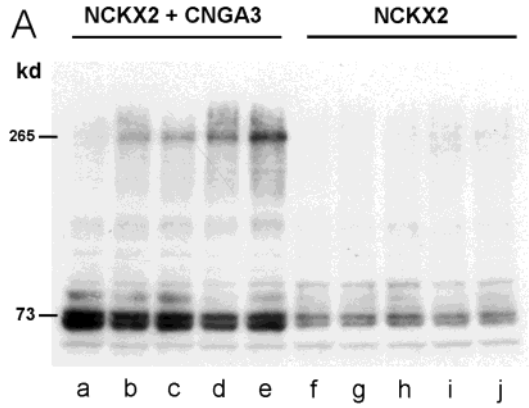

FIGURE 2: Thiol-specific cross-linking of chicken cone NCKX2 containing the Myc epitope. NCKX2 is visualized with an antibody against the Myc epitope. Cells coexpressing the cone NCKX2 and the cone CNGA3 subunit (lanes a−e) or only the cone NCKX2 (lanes f−j) were cross-linked with pPDM for 5 min at 4 °C. Lanes a and f are controls where the cysteines were modified with 10 μM NEM. The pPDM concentrations were the same as in Figure 1, namely, 2.5 μM (lanes b and g), 5 μM (lanes c and h), 7.5 μM (lanes d and i), and 10 μM (lanes e and j). The total protein load was 30 μg per lane.

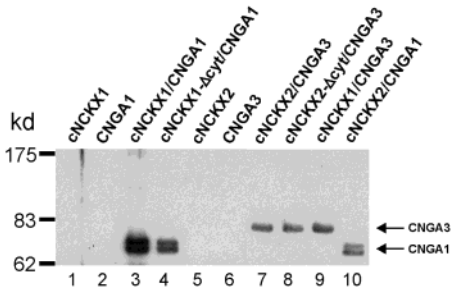

FIGURE 3: Co-immunoprecipitation of 6H2-tagged CNGA subunits with Myc-tagged NCKX. High Five cells were transfected with NCKX and CNGA cDNAs as indicated. Immunoprecipitation was carried out with the Myc polyclonal antibody as described under Materials and Methods. The NCKX proteins contain only the Myc epitope, while CNGA proteins contain only the 6H2 epitope. Samples were separated on SDS−PAGE, transferred to nitrocellulose, and probed with the 6H2 monoclonal antibody.

CNG channel. Therefore, we extended our studies to heterologously expressed cone NCKX2 and the CNGA3 subunit of the cone CNG channel. We used the chicken rod and cone NCKX and CNGA in view of the availability of the appropriate cDNAs, and in view of the fact that both chicken rod and cone NCKX cDNAs give rise to functional NCKX proteins in heterologous systems (*7*).

High Five insect cells were permanently transfected with the cDNA of the Myc-tagged chicken rod NCKX1 and the 6H2-tagged chicken rod CNGA1 subunit (see Materials and Methods) and compared with cells transfected only with the cDNA of the chicken rod NCKX1; the use of different tags permits unambiguous detection of the expressed proteins on Western blots. The effect of the CNGA1 subunit on the efficiency of the NCKX1 cross-linking was examined at 4 °C and at low concentrations of pPDM (0−10 μM). Figure 1 shows that a higher MW band at about 175 kDa appeared for chicken rod NCKX1 as the pPDM concentration was gradually raised to10 μM; this was more pronounced when NCKX1 was coexpressed with CNGA1. This size is consistent with a chicken rod NCKX1 dimer. The Western blot of the CNGA1 subunit showed only the monomer band at 75 kDa (not shown). There was no indication for a cross-link between the CNGA1 subunit and the NCKX or between the CNGA1 subunits themselves. Heterologously expressed CNGA subunits form functional channels and are thought to form tetramers, but apparently no appropriately located cysteine residues were available in chicken rod CNGA1 subunit for thiol-specific cross-linking. In contrast to bovine CNGA1 (*21*), we also did not observe a cross-linked product in the presence of 8Br-cGMP (not shown).

We used the same experimental paradigm to examine a possible interaction between chicken cone NCKX2 and chicken cone CNGA3 subunit (Figure 2). As above, insect cells were transfected either with the cDNAs of cone NCKX2 and cone CNGA3 or with the cDNA of cone NCKX2 alone. The respective epitope tags were the same as described above for rod NCKX1 and rod CNGA1. The chicken NCKX2 monomer invariably showed a doublet (and sometimes a triplet) on SDS−PAGE, of which the lower band represents NCKX2 from which a signal peptide has been cleaved.[2] Thiol-specific cross-linking for 5 min at 4 °C in 0−10 μM pPDM resulted in a distinct NCKX2-containing adduct band at about 265 kDa in cells coexpressing the cone NCKX2 and the cone CNGA3 channel, whereas the adduct band in cells expressing only the cone NCKX2 was almost absent (Figure 2); however, other experiments (see Figure 6, lane 6 and Figure 7, lane 8) clearly show a cross-linked product at about 265 kDa in cells expressing cone NCKX2 alone. The cross-linked product at 265 kDa is considerably higher than the predicted NCKX2 dimer mass. Under our experimental conditions, the cross-link efficiency of cone NCKX2 was consistently greater in the presence than in the absence of the CNGA3 channel, suggesting that the cone CNGA3 subunit interacted with the cone NCKX2. Cross-linking of the cone CNGA3 subunit with NCKX2 shows also an adduct of 265 kDa (not shown). This band is unlikely to be a dimer

[2] Kang and Schnetkamp, submitted.

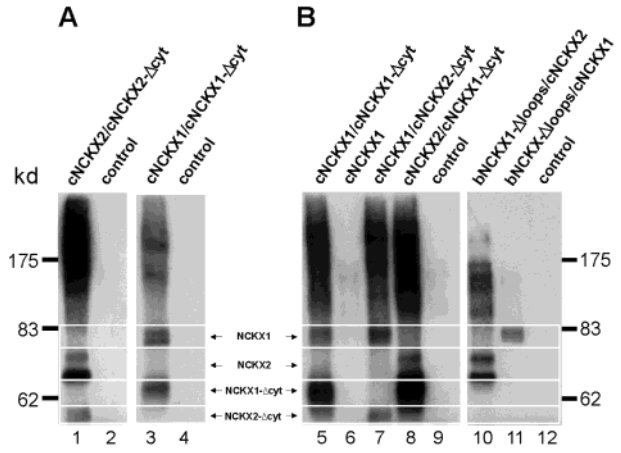

FIGURE 4: Co-immunoprecipitation of Myc-tagged NCKX with 6H2-tagged NCKX. HEK293 cells (A) or High Five cells (B) were transfected with different NCKX cDNAs as indicated. Control indicates nontransfected HEK293 (A) or High Five (B) cells. Immunoprecipitation was carried out with the 6H2 monclonal antibody as described under Materials and Methods. Samples were separated on SDS−PAGE, transferred to nitrocellulose, and probed with the Myc polyclonal antibody.

of two channel subunits because the monomer band is at about 82 kDa, but it might be a cross-linked product between one subunit of CNGA3 with a dimer of NCKX2 (see Discussion).

Co-Immunoprecipitation of CNGA and NCKX. To confirm an interaction between CNG and NCKX, immunoprecipitation of Myc-tagged NCKX was carried with the polyclonal Myc antibody. The immunoprecipitates were tested for the presence of 6H2-tagged CNGA subunits in a Western blot probed with the monoclonal 6H2 antibody. The results illustrated in Figure 3 clearly show that chicken rod CNGA1 was co-immunoprecipitated with chicken rod NCKX1 (lanes 1−3), whereas expression of NCKX1 or CNGA1 separately did not result in the presence of any CNGA1 in the precipitate. Similarly, chicken cone CNGA3 was immunoprecipitated when coexpressed with chicken cone NCKX2 (Figure 3, lanes 5−7).

We made rod and cone NCKX deletion mutants (NCKX−Δcyt) in which the large cytosolic loop was deleted and replaced by a 41 amino acid spacer containing the 6H2 epitope (see Figure 5). Deletion of the cytosolic loop had little effect on either NCKX1 or NCKX2 transport function as assayed by K^+-dependent ^{45}Ca uptake in Na^+-loaded cells (data not shown). Deletion of the large cytosolic loop did not affect the ability of either rod or cone NCKX to pull down CNGA1 (Figure 3, lane 4) or CNGA3 (Figure 3, lane 8), respectively. Finally, lanes 9 and 10 in Figure 3 show that rod NCKX1 could immunoprecipitate cone CNGA3, and conversely, that cone NCKX2 could immunoprecipitate rod CNGA1. The results illustrated in Figure 3 show that heterologously expressed NCKX and CNGA interact. The large cytosolic loop of NCKX appears not critical since deletion of this loop in both rod and cone NCKX did not abolish the NCKX−CNGA interaction. Moreover, no clear isoform specificity was observed as rod NCKX1 could interact with cone CNGA3 and cone NCKX2 could interact with rod CNGA1.

Co-Immunoprecipitation of NCKX1 and NCKX2 Proteins. Cross-linking experiments shown in Figures 1 and 2 suggest that both chicken NCKX1 and NCKX2 are present as oligomers when expressed in High Five cells. Rod NCKX1 appeared to form a dimer, whereas cross-linking cone NCKX2 led to an adduct of a considerably higher MW than that expected for a NCKX2 dimer. This raises the question as to whether NCKX2, like rod NCKX1, forms a homodimer or instead interacts with some other protein. To address an intermolecular association between individual NCKX protein molecules, co-immunoprecipitation experiments were carried out with a monoclonal antibody against the 6H2 epitope. This epitope was contained in the 41 amino acid spacer that replaced the large cytosolic loop in the chicken rod and cone NCKX clones NCKX1−Δcyt and NCKX2−Δcyt (see Figure 5). The first two lanes of Figure 4A show that, when expressed in HEK293 cells, NCKX2−Δcyt could pull down full-length cone NCKX2, while the next two lanes show that NCKX1−Δcyt could pull down full-length NCKX1. A high MW smear was often observed in these experiments, but only in cells transfected with Myc-tagged NCKX. Surface biotinylation only shows the discrete monomer NCKX bands and not the high MW smear (data not shown), suggesting that this smear may be due to not fully mature NCKX proteins that aggregate during the immunoprecipitation protocol. Next, we examined the isoform specificity by expressing different combinations of NCKX cDNAs in High Five cells. As in HEK293 cells, NCKX1−Δcyt could pull down full-length NCKX1 (Figure 4B, lanes 5 and 6). Moreover, NCKX2−Δcyt could pull down full-length NCKX1

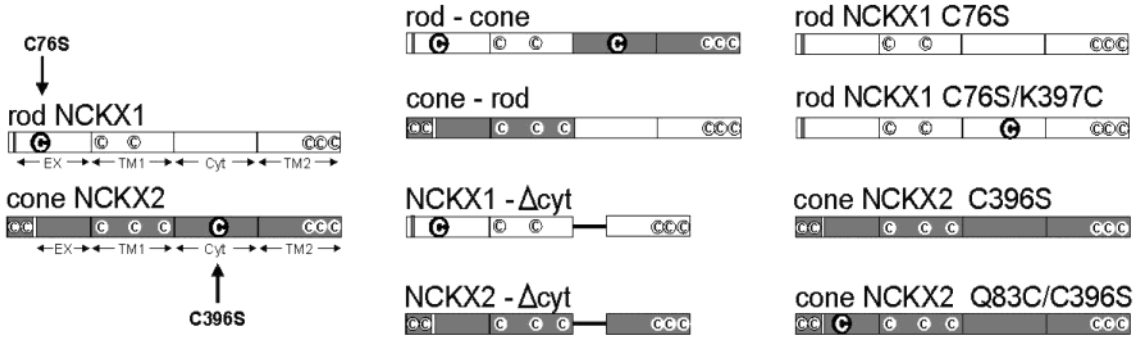

FIGURE 5: Diagram of chicken rod-cone NCKX chimeras. Domain structure of NCKX proteins is presented from left (N-terminus) to right (C-terminus). NCKX proteins are broken up in four domains: (1) signal peptide (indicated by the small bar at the N-terminus) plus extracellular loop at N-terminus, (2) first set of transmembrane spanning segments, (3) large cytosolic loop, and (4) second set of transmembrane spanning segments at C-terminus. The presence of NCKX cysteine residues is indicated with, in bold, the cysteine residues most reactive to the cross-linker. To the right: cysteine mutants used in the experiment described in Figure 7. Residue numbers represent those in the full-length clones as published in ref *7*.

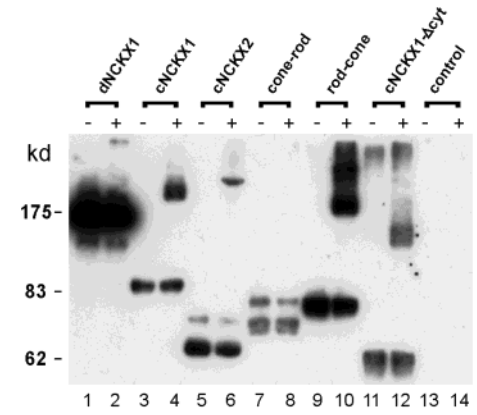

FIGURE 6: Thiol-specific cross-linking of different rod or cone NCKX proteins and chimeras expressed in High Five cells. Western blots, probed with the Myc antibody, are shown of protein extracts from High Five cells expressing the indicated rod or cone NCKX proteins or rod−cone NCKX chimeras. Cells were incubated without cross-linker (−) or with 10 μM pPDM (+) for 10 min on ice as described in Materials and Methods. The total protein load was 20 μg per lane. Control sample represents nontransfected cells.

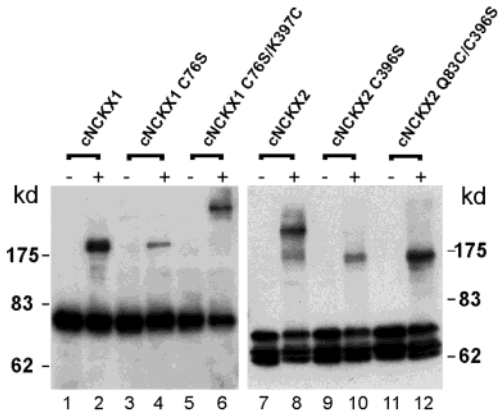

FIGURE 7: Thiol-specific cross-linking of chicken rod and cone NCKX2 in which the cysteine residues in the two large hydrophilic loops are deleted or interchanged. See Figure 5 for the specific location of these cysteine residues. Western blots, probed with the Myc antibody, are shown of protein extracts from High Five cells expressing the indicated rod or cone mutant NCKX proteins. Cells were incubated with 50 μM pPDM for 10 min on ice. The total protein load was 20 μg per lane.

(lane 7), while NCKX1−Δcyt could pull down full-length NCKX2 (lane 8, note that NCKX1−Δcyt and full-length NCKX2 show a very similar MW, except for that NCKX2 always showed an upper band at ~72 kDa). These results suggest that self-assembly of NCKX is not isoform-specific (i.e., NCKX1 could interact with NCKX2), and as observed for the NCKX−CNGA interaction, the large cytosolic loop of the NCKX proteins was not critical in either the NCKX1−NCKX1 interaction or in the NCKX2−NCKX2 interaction.

Earlier, we characterized a bovine rod NCKX1 deletion mutant from which both large hydrophilic loops, the N-terminal loop and the cytosolic loop, were removed (bNCKX1−Δ-loops). The large N-terminal extracellular loop of bovine rod NCKX1 was replaced with that of bovine heart NCX1 (which contains the 6H2 epitope). This double deletion mutant that lacked close to two-thirds of the bovine rod NCKX1 sequence still shows NCKX transport function (*13*). We used this bNCKX1−Δ-loops double deletion mutant to examine a possible role for the N-terminal extracellular loop in NCKX oligomerization. Bovine bNCKX1−Δ-loops was able to pull down both chicken cone cNCKX2 (Figure 4B, lane 10) and chicken rod cNCKX1 (lane 11) (note that the blot probed with the Myc antibody does not show the bNCKX1−Δ-loops construct itself as it lacks the Myc epitope). The combined results with the use of the different deletion constructs suggest that the intermolecular NCKX−NCKX interaction is not critically dependent on either of the two large hydrophilic loops.

Co-Immunoprecipitation of NCKX1 and NCKX2 Proteins. The experiments illustrated in Figures 1 and 2 show a distinct pattern of cross-linking for rod and cone NCKX, respectively. Rod NCKX1 appears to cross-link to a dimer, whereas cross-linking of cone NCKX2 leads to a higher MW adduct. We have made chicken rod−cone NCKX chimeras to determine the NCKX domain responsible for the distinct cross-link pattern observed for NCKX1 and NCKX2, respectively. Figure 5 illustrates the different rod−cone NCKX chimeras and deletion mutants used; since we use a thiol-specific cross-linker, the positions of all the cysteine residues found in chicken NCKX1 and NCKX2 are indicated as well.

In addition to the short chicken rod and cone NCKX clones (~650 residues), we also used the dolphin rod dNCKX1 (*6*) to represent the much larger mammalian rod NCKX1 clones (>1000 residues) since in situ NCKX dimerization has only been studied for bovine rod NCKX1 (*19*, *21*). The first two lanes in Figure 6 show that a high MW adduct could be observed with the dolphin rod NCKX1, but clearly at a much lower cross-link efficiency as compared with the smaller chicken rod and cone NCKX. The next four lanes (Figure 6, lanes 3−6) show the distinctly sized cross-link products obtained with the unmodified chicken rod NCKX1 and cone NCKX2 proteins, respectively. Interestingly, the CONE−ROD NCKX chimera showed no cross-link products, whereas the ROD−CONE NCKX chimera showed two distinct cross-link products. Allowing for the differences in MW of the monomers, the positions of the cross-linked products observed for the ROD−CONE chimera were similar to those of the distinct adduct products observed for NCKX1 and NCKX2 individually. Examination of the location of the cysteine residues in the chicken rod and cone NCKX sequences (Figure 5), respectively, reveals that, in addition to highly conserved cysteine residues in the transmembrane spanning segments, cone NCKX2 has a single unique cysteine residue in the large cytosolic loop, while rod NCKX1 has a single unique cysteine in the N-terminal external loop (these two cysteine residues are presented in bold in Figure 5). The CONE−ROD chimera, which showed no cross-linked products, lacks the cysteine residue located in the middle of the cytosolic loop of NCKX2 and the cysteine residue located in the N-terminal extracellular loop of NCKX1. Conversely, the ROD−CONE chimera contains both these cysteine residues and showed two distinct adduct products.

The above observations suggest a simple pattern for cross-linking: the cysteine residue in the middle of the extracellular loop of NCKX1 permits formation of a dimer, whereas the cysteine residue in the middle of the large cytosolic loop of NCKX2 results mainly in an adduct that runs at a higher MW than expected for a dimer. The cysteine residues in the transmembrane spanning segments did hardly react with the cross-linker under our experimental conditions.

Cross-Link Pattern Depends on Location of Cysteine Residue, Not on NCKX Isoform. To examine whether the

nature of the adduct band observed for rod NCKX1 and cone NCKX2, respectively, was specific for the NCKX isoform or merely reflected the location of an appropriately located reactive cysteine residue, we altered the location of this reactive cysteine residue in both rod NCKX1 and cone NCKX2, respectively. Figure 7 illustrates that mutation of the extracellular Cys76Ser greatly reduced the amount of NCKX1 dimer observed, whereas mutation of Cys76Ser combined with insertion of a cysteine in the large cytosolic loop (the Cys76Ser−Lys397Cys double mutant) led to a cross-linked product significantly higher than that of the dimer observed for wild-type chicken rod NCKX1 (i.e., the phenotype observed for wild-type cone NCKX2). Conversely, mutation of the cytoplasmic Cys396Ser in cone NCKX2 abolished the high MW adduct band observed for chicken cone NCKX2, whereas mutation of Cys396Ser combined with insertion of a cysteine in the N-terminal extracellular loop (the Cys396Ser−Gln83Cys double mutant) led to a cross-linked product of a much reduced apparent MW consistent with an NCKX2 dimer (i.e., the phenotype of wild-type rod NCKX1). In the experiment illustrated in Figure 7, a higher concentration of 50 μM pPDM was used, and some cross-linking to dimers was observed in the absence of the more reactive cysteine residues in the middle of the large hydrophilic loops. This suggests that cysteine residues in the transmembrane spanning domains can result in cross-linking albeit with much lower efficiency. In conclusion, the apparent molecular mass of the cross-linked product is related to the location of the cysteine residue utilized by the cross-linker and does not appear to depend on the surrounding sequence (i.e., that of NCXK1 or that of NCKX2). The presence of a cysteine residue in the N-terminal extracellular loop leads to an adduct band consistent with a NCKX dimer; the presence of a cysteine residue in the large intracellular loop leads to an adduct band of an apparent MW considerably higher than that expected for a dimer, independent of the specific sequence in which the cysteine residue is present.

DISCUSSION

Association of NCKX and CNGA. Previous studies have shown that the bovine rod Na^+/Ca^{2+}-K^+ exchanger (NCKX1) exists as a dimer incorporated into a larger complex with the cGMP-gated channel (CNG) and other proteins in rod outer segment membranes (*20*−*22*). Recent experimental evidence indicates that the rod CNG channels consist of three CNGA1 subunits and one CNGB1 subunit (*23*−*25*). The CNGA1, but not the CNGB1 subunit, has been shown to interact with NCKX1 in bovine rod photoreceptors, and it has been suggested that CNGA1 facilitates the self-assembly of bovine rod NCKX1 to form a dimer (*21*). Here, we present the first demonstration that heterologously expressed NCKX proteins interact with the CNGA1 subunit. Both rod NCKX1 and cone NCKX2 interacted with their respective CNGA1 and CNGA3 partners, while rod NCKX1 could interact with cone CNGA3, and cone NCKX2 could interact with rod CNGA1. Indirect evidence for the NCKX−CNGA interaction was obtained by the observation that coexpression of CNGA and NCKX facilitated thiol-based cross-linking of NCKX into homo-oligomers (Figures 1 and 2), while more direct evidence was obtained by co-immunoprecipitation experiments (Figure 3). These results show that the NCKX−CNG interaction is not limited to the large bovine rod NCKX1 protein (1216 residues) but also occurs with the much smaller chicken rod NCKX1 and cone NCKX2 proteins (~650−660 residues). Our results strongly suggest that interactions between CNGA and NCKX are common in cells that coexpress these two proteins.

Rod NCKX1 and Cone NCKX2 Each Form Homooligomers. Although coexpression of CNGA and NCKX led to a facilitation of NCKX oligomerization, cross-linking was readily observed in the absence the CNGA subunit, and the adduct bands had similar MW as those observed in the presence of CNGA (Figures 1, 2, and 6). Application of the thiol-specific cross-linker pPDM clearly led to the appearance of higher MW adducts for both chicken rod and cone NCKX, as well as for dolphin rod NCKX1, although in the latter case the cross-linking efficiency was much lower (Figure 6), similar to the finding reported earlier for bovine NCKX1 (*19*). However, most of the NCKX protein was not cross-linked under our rather stringent conditions with low cross-linker concentrations. This raises the question as to whether only a minor fraction of the NCKX molecules is present as oligomers. When cross-linking was carried out in membrane vesicles isolated from intact cells, a much higher cross-link efficiency was observed with much less (NCKX1) or very little (NCKX2) monomer left (data not shown). This suggests that heterologously expressed NCKX1 and NCKX2 occur predominantly as oligomers. The lower cross-link efficiency observed in intact cells was likely due to the abundance of cytosolic proteins and the presence of low MW substances such as glutathione that consume most of the cross-linker. The occurrence of homo-oligomers for both rod NCKX1 and cone NCKX2 was corroborated by the co-immunoprecipitation experiments shown in Figure 4.

At the low cross-linker concentrations used here, cross-linking of rod NCKX1 yielded a high MW adduct consistent with a dimer, whereas cross-linking of cone NCKX2 led mainly to an adduct with a MW considerably higher than expected for a dimer. This finding was independent of the presence of CNGA3 (Figures 2 and 6). Experiments with rod−cone NCKX chimeras (Figure 6) and experiments with mutants in which the position of the cysteine residues in the large hydrophilic loops was altered (Figure 7) indicated that the cross-link phenotype was actually dependent on the location of a single cysteine residue rather than dependent on the surrounding sequence (i.e., NCKX1 or NCKX2). Both NCKX1 and NCKX2 were cross-linked to dimers if the reactive cysteine was present in the N-terminal extracellular loop. As only a single reactive cysteine appeared to be mainly accessible to the cross-link reagent in this case, thiol-based cross-linking can only yield a dimer, but our results do not preclude that NCKX may form trimers or tetramers. It should be pointed out that thiol-specific cross-linking requires the presence of appropriately placed cysteine residues, but this does not imply that these cysteine residues are important in mediating the intermolecular NCKX−NCKX interaction per se.

Nature of the High MW NCKX Cross-Link Product. When the reactive cysteine was placed in the large cytosolic loop, both NCKX1 and NCKX2 yielded a cross-link product with a MW considerably higher than that expected for a dimer (Figure 7). Three interpretations of this result may be considered. First, the NCKX dimer cross-linked via the

cytosolic domain can associate quickly with another NCKX monomer to form a trimer; second, both NCKX1 and NCKX2 associate with another cellular protein and can be cross-linked to this protein but only when a reactive cysteine is present in the middle of the cytosolic loop; finally, the NCKX dimer cross-linked via the cytosolic domain cannot unfold properly in the SDS-sample buffer and runs at an anomalously high MW on SDS−PAGE. Intriguingly, when insect cells coexpressed NCKX2 and CNGA3, cross-linking yielded an adduct band of CNGA3 at about 265 kDa suggesting perhaps the presence of a NCKX2 dimer with a CNGA3 molecule (not shown). Further studies are needed to resolve this issue. Moreover, it should be noted that both rod and cone CNG channels were recently shown to consist of three CNGA1 subunits and one CNGB1 subunit (*23*−*25*). As NCKX interacts with CNGA (Figure 3), it is quite conceivable that three NCKX molecules bind to the CNG channel.

Specificity of the NCKX−NCKX and NCKX−CNGA Interactions. Cross-linking experiments with rod cone NCKX1−NCKX2 chimeras revealed cross-link products as long as a reactive cysteine residue was present (Figure 6), suggesting that the interaction was not isoform specific. This inference was further confirmed by co-immunoprecipitation experiments with NCKX molecules containing different tags as illustrated in Figure 4. Thus, cone NCKX2 could co-immunoprecipitate with rod NCKX1 and vice versa. Consistent with this finding, the NCKX−NCKX interaction appeared to be mediated by the highly conserved transmembrane spanning segments as the bovine rod NCKX1−Δloops construct that lacked the two large hydrophilic loops could still pull down both rod NCKX1 and cone NCKX2 (Figure 4B). The two large hydrophilic loops are the least conserved sequence elements in the NCKX sequence and appear not to be critically involved in NCKX oligomerization even though these large hydrophilic loops of individual NCKX molecules come into close contact as evidenced by the efficient cross-linking mediated by cysteine residues located in these loops.

Similar to the NCKX1−NCKX2 interaction, the interaction between CNGA and NCKX was not isoform specific (i.e., rod NCKX1 could interact with cone CNGA3, and cone NCKX2 could interact with rod CNGA1 (Figure 3)). The CNGA−NCKX interaction was not critically dependent on the large cytosolic loop of NCKX, as observed for NCKX self-assembly. These findings suggest that both NCKX−NCKX and CNGA−NCKX interactions are predominantly mediated by the hydrophobic transmembrane spanning segments.

Physiological Implications. Protein complexes are often of paramount importance for proper physiological function. In bovine rod photoreceptors, the CNG−NCKX complex is only present in the plasma membrane of the outer segment organelle (*28*), and this complex interacts with two other proteins present in the rims of the intracellular disk membranes (*22*). This rather specific arrangement may not be found elsewhere, and hence, the more general occurrence of NCKX−CNG interaction, even in cone photoreceptors, needed to be determined. The results reported here strongly suggest that the interaction between NCKX and the CNGA subunit of the CNG channel is found not only in rod photoreceptors of other species than bovine but also in cone photoreceptors. Our results also suggest that both NCKX−NCKX and NCKX−CNGA interactions may be general phenomena shared by different NCKX and CNGA isoforms expressed in cells other than photoreceptors. The ability of CNG channels to mediate a sustained depolarizing current carried in part by Ca^{2+} may in fact require the presence of NCKX for proper Ca^{2+} extrusion; Ca^{2+} extrusion via the K^+-independent Na^+/Ca^{2+} would be unfavorable because the sustained depolarized membrane potential of rod and cone photoreceptors that exists in darkness would compromise Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger operating at a stoichiometry of $3Na^+$:$1Ca^{2+}$ (*29*). In the outer segments of photoreceptors, the CNG channels constitute the only source for Ca^{2+} influx, whereas NCKX is the only mechanism for Ca^{2+} extrusion. Therefore, CNG channels and NCKX are the only proteins in the outer segments of photoreceptors that control Ca^{2+} homeostasis. Co-localization of these two proteins may have several implications: (1) a direct regulatory interaction between channel and exchanger may exist; indirect evidence for such a mechanism has recently been reported (*30*); (2) intracellar Ca^{2+} diffusion may be largely restricted to the immediate vicinity of the NCKX−CNG channel complex providing for local Ca^{2+} signaling; modeling of Ca^{2+} diffusion suggests that a significant fraction of the Ca^{2+} entering the outer segment can in fact be localized by this mechanism (*31*); (3) the NCKX−CNG interaction may be required to regulate protein synthesis and establish a fixed molar ratio of both proteins in the cell as this ratio critically controls cytosolic free Ca^{2+} concentration in photoreceptors in darkness. Demonstration of heterologously expressed CNGA−NCKX complexes, as shown in this study, should enable further studies on the functional and structural consequences of the NCKX−CNGA interaction.

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