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Binding of the cGMP-gated Channel to the Na/Ca-K Exchanger in Rod Photoreceptors*

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The intracellular Ca^{2+} concentration in rod outer segments of vertebrate photoreceptors is controlled by Ca^{2+} influx through cGMP-gated channels and by Ca^{2+} efflux driven by Na/Ca-K exchangers. Previously, we suggested that channel and exchanger are associated (Bauer, P. J., and Drechsler, M. (1992) *J. Physiol. (Lond.)* 451, 109–131). This suggestion has been thoroughly examined using a variety of biochemical approaches. First, we took advantage of the fact that cGMP-gated channels bind calmodulin (CaM). Using CaM affinity chromatographic purification of the channel in 10 mM CHAPS, a significant fraction of exchanger was co-eluted with the channel indicating a binding affinity between channel and exchanger. Binding of channel and exchanger was examined more directly by cross-linking of proteins in the rod outer segment membranes. Activation of the channel with cyclic 8-bromo-GMP lead to exposure of a cysteine, which allowed cross-linking of the channel to the exchanger with the thiol-specific reagent DL-1,4-bismaleimido-2,3-butanediol. Cleavage of the cross-links and electrophoretic analysis indicated that a cross-link between the α-subunit of the channel and the exchanger formed. Furthermore, a cross-link between two adjacent α-subunits of the channel was found, suggesting that the α-subunits of the native channel are dimerized. Further support for an interaction between α-subunit and exchanger was obtained by *in vitro* experiments. Specific binding of the exchanger to the α-subunit but not to the β-subunit of the channel was observed in Western blots of purified channel incubated with purified exchanger. This study suggests that two exchanger molecules bind to one cGMP-gated channel and, more specifically, that binding of exchanger molecules occurs at the α-subunits, which in the native channel are dimerized. The implications of these findings regarding the possibility of local Ca^{2+} signaling in vertebrate photoreceptors will be discussed.

In the dark, a steady cation influx enters the vertebrate photoreceptors through the cGMP-gated channels (dark current), which depolarizes the cell to about −40 mV (see *e.g.* Refs. 2 and 3). Whereas most of this dark current in rod photoreceptors is carried by Na^+ ions, about 15% of the current is because of influx of Ca^{2+} ions, and about 5% is because of an influx of Mg^{2+} ions in rod photoreceptors (4). Photon absorption by rhodopsin activates the visual cGMP cascade, which leads to rapid hydrolysis of cGMP to GMP, thus closing the channels and hyperpolarizing the cell (reviewed in Refs. 5 and 6).

Na^+ ions are primarily responsible for the light-induced voltage signal of the photoreceptor, whereas Ca^{2+} ions function intracellularly as a second messenger in sensitivity regulation and recovery. Ca^{2+} is extruded out of the cell by a Na/Ca-K exchanger, which utilizes both the Na^+ inward and the K^+ outward gradient as driving forces for the uphill Ca^{2+} transport (7, 8). Closure of the cGMP-gated channels upon photon excitation of the cell leads to a drop in the internal Ca^{2+} concentration (9–11). This negative Ca^{2+} signal plays a key role in light adaptation of photoreceptors (12–16). If locally excited by a flash of light, adaptation in rod outer segment is a spatially restricted phenomenon (17). Very recently, it has been shown that the spatial spread of adaptation upon excitation of a rod photoreceptor by two photon absorption and the intracellular Ca^{2+} diffusion kinetics is largely consistent with the hypothesis that Ca^{2+} is a messenger of adaptation (18).

In several neuronal and neurosecretory cells, Ca^{2+} signaling has been established to be a local event occurring only in very restricted microdomains (19–23). For rod photoreceptors, we have recently demonstrated that Na/Ca-K exchangers form dimers in the plasma membrane of the rod outer segment (24). Moreover, we suggested from a Ca^{2+} flux study that exchanger and channel are associated indicating that possibly in photoreceptors, too, Ca^{2+} signaling may be restricted to the close vicinity of the channel (1). We report here biochemical evidence supporting the idea that the Na/Ca-K exchanger is bound to the cGMP-gated channel in the plasma membrane of rod photoreceptors. Short reports of this study have been given (25, 26).

MATERIALS AND METHODS

Preparation of Bovine ROS Membranes—Purified ROS[1] were prepared by discontinuous sucrose flotation as described (27). Aliquots of the ROS that contained 5 mg of rhodopsin were pelleted in 600 mM sucrose for 30 min at 15,000 × *g* at 4 °C (buffer: 10 mM HEPES adjusted to pH 7.0 with KOH, 300 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol), shock-frozen in liquid nitrogen, and stored at −80 °C until use. ROS membranes were prepared by hypotonic lysis of purified ROS in 0.5 mM Tris, 0.5 mM HEPES adjusted to pH 7.4, 1 mM EDTA, and the protease inhibitors 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 2.2 μg/ml *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

Cross-linking and Protein Modification—To avoid bleaching of rhodopsin, cross-linking with ROS membranes was carried out in dim red light. Cross-linking with the dimaleimide BMDB[2] and modification of cysteine groups with NEM were carried out as described previously (24). Biotinylation of cysteines was carried out by incubation of the ROS

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[1] The abbreviations used are: ROS, rod outer segment; BMDB, DL-1,4-bismaleimido-2,3-butanediol; NEM, *N*-ethylmaleimide; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate; PC, L-α-phosphatidylcholine; 8-Br-cGMP, cyclic 8-bromo-GMP; CaM, calmodulin.

[2] This is identical to the recently described reagent BMBD (24); this reagent is now commercially available from Pierce, Calbiochem, or Fluka.

membranes at room temperature in 200 μM biotinmaleimide (buffer: 10 mM HEPES/NaOH, pH 6.5, 100 mM NaCl, 1 mM EDTA). The reaction was terminated after 15 min by adding 10 mM dithiothreitol (final concentration).

CaM Affinity Chromatography—Purification of the cGMP-gated channel and of the thiol-specific cross-links of the channel was carried out by CaM affinity chromatography following the protocol of Hsu and Molday (28) with some modifications. ROS membranes (containing 30 mg of rhodopsin) were solubilized in 30 ml of 18 mM CHAPS (buffer: 10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 1 mM dithiothreitol) for 30 min at 4 °C and centrifuged for 30 min at 48,300 × *g*. The supernatant was loaded on 4 ml of CaM-agarose (Sigma, P-4385), and nonbound proteins were eluted with ~15 ml of 15 mM CHAPS in the same buffer but with 200 mM NaCl and 1 mM $CaCl_2$ (wash buffer). Bound proteins were eluted with 15 mM CHAPS in the same buffer but with 150 mM NaCl and with 2 mM EDTA instead of $CaCl_2$ (elution buffer).

Functional Reconstitution into PC Vesicles of Purified cGMP-gated Channel—To purify the cGMP-gated channel by CaM affinity chromatography for functional reconstitution into PC vesicles, 2 mg/ml PC was present throughout, and the $CaCl_2$ concentration was 0.5 mM in the solubilization and the wash buffers. Moreover, a significant fraction of exchanger was co-purified if the CHAPS concentration was lowered to 10 mM throughout the CaM affinity chromatography (see Fig. 1). Functional reconstitution was carried out as described in Ref. 29 with some modifications. Briefly, final concentrations of 10 mg/ml PC and 10 mM $CaCl_2$ were added to the bound fraction, and the solution was adjusted to 100 mM KCl, 10 mM CHAPS. The detergent was removed by dialysis for 20 h at 4 °C against 4 liters of 5 mM HEPES/KOH, pH 7.4, 100 mM KCl, 2 mM $CaCl_2$, 0.1 mM dithiothreitol with one change of the dialysis buffer.

Co-elution of cGMP-gated Channel and Exchanger from a CaM Affinity Column—To examine the binding affinity between the solubilized exchanger and the cGMP-gated channel, the solubilization and purification by CaM chromatography was carried out in 10 mM CHAPS and 1 mg/ml PC, leaving the other conditions unchanged (see "CaM Affinity Chromatography"). After the addition of 12 mM $CaCl_2$, the bound fraction was subjected to a second CaM affinity chromatography.

Ca^{2+} Flux Assay of Ca^{2+}-loaded PC Vesicles—External Ca^{2+} was removed by passage of the suspension through Chelex 100 in the K-form (Bio-Rad) using a Swinnex-13 filter holder (Millipore) with a 3-μm pore size Nuclepore filter. Samples of 1 ml were added to a stirring cuvette (OS119–004, Hellma, Müllheim, Germany), and 50 μM arsenazo III were added. Changes in the Ca^{2+} concentration were monitored as absorption difference between 652 and 700 nm in a dual wavelength spectrophotometer (Aminco DW-2000; SLM Instruments, Urbana, IL). Ca^{2+} calibration and determination of initial Ca^{2+} fluxes was carried out as described previously (30).

Binding of the Exchanger to Purified and Blotted cGMP-gated Channel—After biotinylation of ROS membranes (see above), the exchanger was purified in three consecutive chromatographic steps: by DEAE cellulose ion exchange, by AF red affinity, and by concanavalin A-Sepharose affinity chromatography, as described (29, 31). A Western blot of the cGMP-gated channel, purified as described above, was incubated for 16 h at 4 °C in phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Nonspecific binding sites were blocked with 1% (w/v) nonfat milk and 0.02% Tween-20 in phosphate-buffered saline and incubated to purified biotinylated exchanger in phosphate-buffered saline and 0.02% Tween-20 for 1 h. After thorough washings, this blot was labeled either with streptavidin coupled to horseradish peroxidase or with antibodies against the exchanger or against α- or β-subunit of the channel. Streptavidin, but not the exchanger molecules, could be removed from the blot by the same procedure as the antibodies (*i.e.* by incubation for 45 min at 65 °C in 2% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 62 mM Tris/HCl, pH 6.8) thus allowing the same blot to be labeled several times (24).

Immunoblotting and Protein Analysis—Electrophoresis on a linear 3.5–7.5% gradient polyacrylamide gel and Western blotting were performed as described previously (24). Protein content was determined following the Amido Black procedure (32) using filters of nitrocellulose 0.45-μm pore size (HATF, Millipore).

RESULTS

Simultaneous Elution of Channel and Exchanger—Based on a Ca^{2+} flux study, we suggested in a previous report that the cGMP-gated channel and the Na/Ca-K exchanger are associated in the plasma membrane of rod photoreceptors (1). To examine this suggestion, we asked if under certain conditions this association persists after solubilization in detergent. If so, then we should expect that extraction of the channel by affinity chromatography would result in a selective enrichment of exchanger.

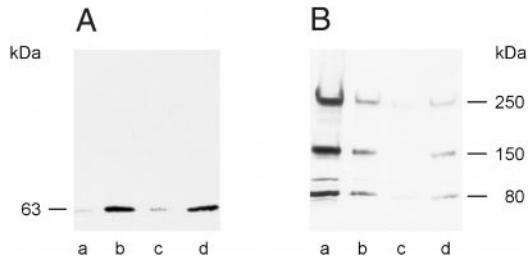

FIG. 1. **Co-elution of cGMP-gated channel and Na/Ca-K exchanger from a CaM affinity column.** ROS membranes solubilized in 10 mM CHAPS (see "Materials and Methods") were subjected to CaM affinity chromatography. The bound fraction was eluted with 2 mM EDTA and, after addition of 12 mM $CaCl_2$ subjected to a second CaM affinity chromatography. The nonbound and the bound fraction after each chromatography step were analyzed by SDS-polyacrylamide gel electrophoresis on a 3.5–7.5% polyacrylamide gel and blotted; nonbound fractions of the first and second chromatography are shown in *lanes a* and *c*, respectively; bound fractions are shown in *lanes b* and *d*, respectively. The same amount of bound protein (1.8 μg) was loaded on *lanes b* and *d*, and the volume taken of the nonbound fraction was the same as that of the bound fraction in each chromatography. The Western blot was consecutively labeled with monoclonal antibodies PMc 2G11 against the α-subunit of the channel (*A*) and PMe 1B3 against the exchanger (*B*).

In agreement with an earlier report (28), we found that cGMP-gated channels purified by CaM-agarose affinity chromatography contained virtually no exchanger if ROS membranes were solubilized in 18 mM CHAPS and 200 mM NaCl (not shown). However, a significant amount of exchanger was detected in the bound fraction of the CaM-agarose column if the detergent and the salt concentration were lowered to 10 mM CHAPS and 100 mM NaCl, as shown in Fig. 1. Although the nonbound protein fraction contained most of the exchanger and some channel protein, the bound channel was eluted together with a significant amount of exchanger (Fig. 1, *lanes a* and *b*). The fractions collected immediately before elution of the bound proteins (*i.e.* after a 15-ml wash period) did not contain any detectable amount of channel or exchanger (not shown). Even a second CaM affinity chromatography of the channel did not completely remove the exchanger (Fig. 1, *lane d*). Because highly purified exchanger did not bind on a CaM-agarose column (data not shown), this observation supports our hypothesis that the exchanger interacted with the channel protein.

The presence of exchanger in cGMP-gated channel eluted from a CaM affinity column was also evident from Ca^{2+} flux studies. ROS membranes were solubilized in 10 or 15 mM CHAPS; the cGMP-gated channel was extracted by CaM affinity chromatography and reconstituted in the presence of Ca^{2+} into PC vesicles by dialysis to remove the detergent. If these vesicle preparations were assayed for Na^+- and cGMP-induced Ca^{2+} fluxes, there was very little Na^+-induced Ca^{2+} flux in vesicles prepared with channel, which was solubilized and eluted at 15 mM CHAPS (Fig. 2, *curve a*), but there was a substantial Na^+-induced Ca^{2+} flux in vesicles prepared with channel that was solubilized and eluted at 10 mM CHAPS (Fig. 2, *curve b*); the cGMP-induced Ca^{2+} fluxes were similar in both preparations.

Thiol-specific Cross-linking—To examine more directly if the cGMP-gated channel and Na/Ca-K exchanger are associated in the rod photoreceptor plasma membrane, chemical cross-linking of adjacent proteins was used. We used the novel cleavable

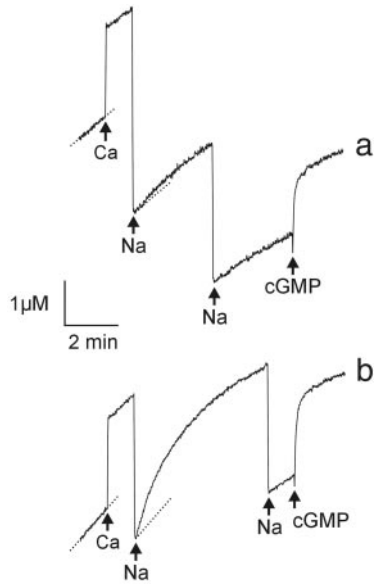

FIG. 2. **Ca^{2+} fluxes from proteoliposomes containing a cGMP-gated channel eluted from a CaM affinity column.** cGMP-gated channels were extracted by CaM affinity chromatography from ROS membranes solubilized in CHAPS and reconstituted into PC vesicles. Reconstitution was carried out by adding 10 mg/ml PC and removal of detergent by dialysis overnight. Two different detergent concentrations were used to extract the channel: 15 mM CHAPS (*a*) and 10 mM CHAPS (*b*). Ca^{2+} concentration was monitored spectroscopically with 50 μM arsenazo III in the dual wavelength mode (OD 652–700 nm). Additions at the indicated times were (final concentrations): 2 μM $CaCl_2$, 95 mM NaCl, 181 mM NaCl, and 180 μM cGMP. The recordings were scaled to indicate Ca^{2+} concentration changes using the initial calibrating 2-μM Ca^{2+} pulse. The passive Ca^{2+} leakages, indicated by *dotted lines*, were 0.518 μM/min and 0.696 μM/min in the recordings *a* and *b*, respectively (downward deflections upon Na^+ additions were because of Na^+-dependent decreases of the Ca^{2+} affinity of arsenazo III; the amplitudes of this effect correlated with the fractions of Ca^{2+}-bound arsenazo III).

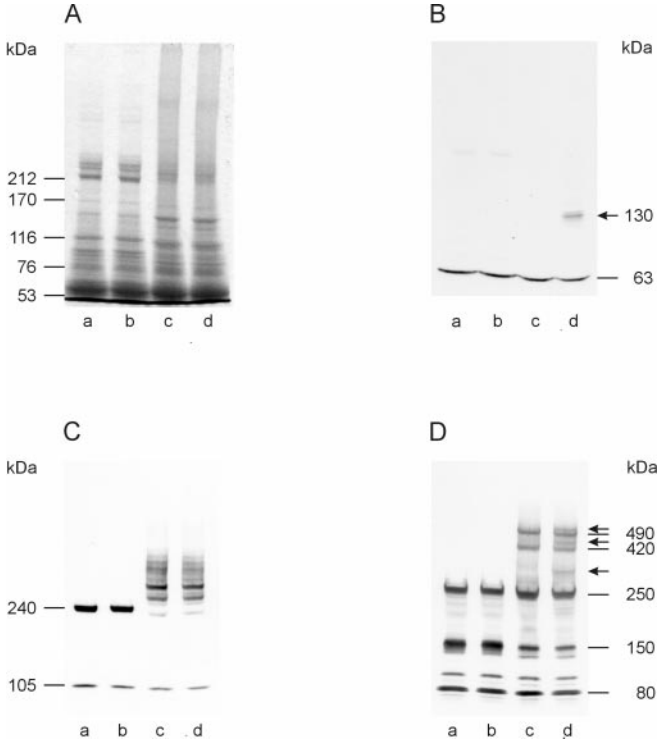

FIG. 3. **Thiol-specific cross-linking of the cGMP-gated channel and Na/Ca-K exchanger in ROS membranes.** ROS membranes prepared from purified ROS by hypotonic washes and cross-linked with the dimaleimide BMDB either in the absence (*lane c*) or presence of 8-Br-cGMP (*lane d*). Before cross-linking, washed ROS membranes are shown without (*lane a*) and after the addition of 50 μM 8-Br-cGMP (*lane b*). Electrophoresis was carried out in 5% β-mercaptoethanol on a 3.5–7.5% polyacrylamide gel with 70 μg of protein in each lane. Shown are a Coomassie Blue-stained gel (*A*) and a Western blot that was consecutively labeled with the monoclonal antibody against the α-subunit (PMc 2G11; *B*), the polyclonal antibody against the β-subunit (PPc 32K; *C*) of the channel (42), and the monoclonal antibody against the exchanger (PMe 1B3; *D*). The *arrows* in *D* point to cross-linking bands of the Na/Ca-K exchanger at 330, 465, and 545 kDa which appear only if cross-linking has been carried out in the presence of 8-Br-cGMP.

dimaleimide reagent BMDB, which cross-links adjacent cysteines (24). Fig. 3 shows a Western blot of rod outer segment membranes upon cross-linking with the dimaleimide BMDB in the absence (*lane c*) and presence of 8-Br-cGMP (*lane d*); as a control, *lanes a* and *b* show the non-cross-linked membranes in the absence and presence of 8-Br-cGMP, respectively. The same Western blot has been consecutively labeled with antibodies against the α-subunit (*B*) and the β-subunit (*C*) of the channel and with antibodies against the Na/Ca-K exchanger (*D*). Between successive labelings, the antibodies had been removed from the blotting membrane, as described under "Materials and Methods."

If cross-linking with BMDB was carried out in the absence of 8-Br-cGMP, no cross-linking band of the α-subunit of the channel was labeled (Fig. 3*B*, *lane c*). In contrast, the β-subunit of the channel was almost quantitatively cross-linked to other proteins (*C*, *lane c*). The numerous cross-linking bands of the β-subunit have not been analyzed in detail; however, these bands were not because of cross-links between the β-subunit and exchanger, because the exchanger cannot be cross-linked to the channel under these conditions (24). These bands were also not because of cross-links between the β-subunit and the α-subunit of the cGMP-gated channel, as will be shown below. The weak band at about 105 kDa in Fig. 3*C* was presumably because of the β′-subunit, which is the β-subunit lacking the cytoplasmic glutamic acid-rich protein domain. The cross-linking bands of the exchanger at 490 and 420 kDa (*D*, *lane c*) have been thoroughly studied previously; the 490-kDa band was due to the homodimer of the exchanger, and the 420-kDa band was due to the cross-link of the exchanger with a 150-kDa exchanger fragment (24).

In the presence of 8-Br-cGMP, a distinct cross-linking band of the α-subunit was visible at about 130 kDa, *i.e.* at about twice the molecular mass of the monomeric α-subunit (Fig. 3*B*, *lane d*; non-cross-linked control: *lane b*). On the other hand, the cross-linking band pattern of the β-subunit showed no obvious cGMP-dependent change (Fig. 3*C*, *lanes c* and *d*). In contrast, comparing the cross-linking bands of the exchanger obtained in the presence of 8-Br-cGMP with the cross-linking bands obtained in the absence of 8-Br-cGMP (*i.e. lane d* with *c* in *D*) revealed three additional bands in *lane d* centered at about 330, 465, and 545 kDa (*arrowheads*). Although these additional cross-linking bands were weak, they were consistently found if cross-linking of ROS membranes had been carried out with dimaleimides in the presence of 8-Br-cGMP.

This experiment suggested (*a*) that upon 8-Br-cGMP binding to the channel at least one cysteine of the channel became accessible to thiol-specific reagents, which was not accessible in the absence of 8-Br-cGMP and (*b*) that cross-links between the cGMP-gated channel and exchanger formed that were not labeled with the antibodies against the α- or β-subunits of the channel. Both suggestions will be investigated below.

Binding of 8-Br-cGMP to the Channel Exposes a Cysteine—To examine the first presumption, the experiment was extended in the following way. Before cross-linking, the accessible cysteines were blocked with NEM either in the absence (Fig. 4, *lane b*) or in the presence (Fig. 4, *lane c*) of 8-Br-cGMP. Then, excess reagent was thoroughly washed off, and the pro-

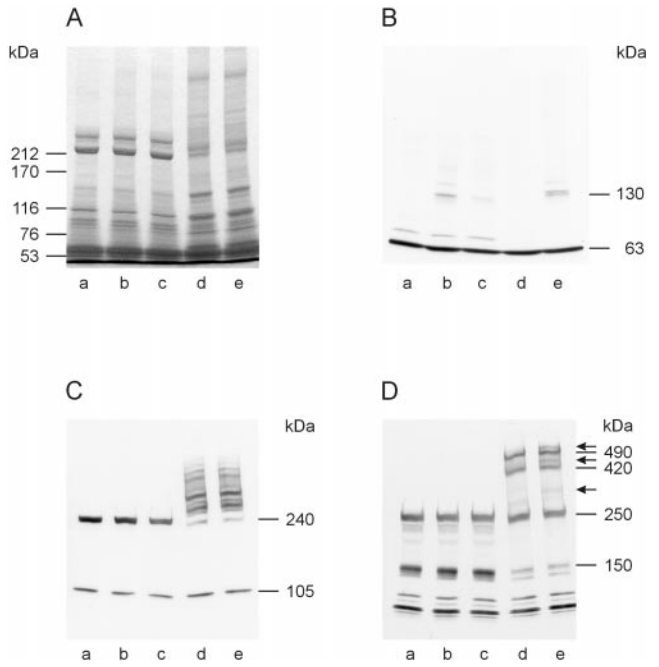

FIG. 4. **Reactive cysteine for cross-linking of the channel α-subunit not accessible to NEM in the absence of 8-Br-cGMP.** Before cross-linking with BMDB in the presence of 50 μM 8-Br-cGMP, ROS membranes were modified with NEM either in the absence (*lane b*) or presence of 50 μM 8-Br-cGMP (*lane c*). For comparison, non-NEM-treated ROS membranes are shown before (*lane a*) and after cross-linking with BMDB either in the absence (*lane d*) or presence of 50 μM 8-Br-cGMP (*lane e*). Electrophoresis is as in Fig. 3 with 70 μg of protein in each lane. Coomassie Blue-stained gel (*A*) and a Western blot were reprobed with antibodies against the α-subunit (*B*) and the β-subunit of the channel (*C*) and with antibodies against the exchanger (*D*). Antibodies are the same as in Fig. 3. The *arrows* in *D* point to the cGMP-dependent cross-linking bands mentioned in the legend to Fig. 3.

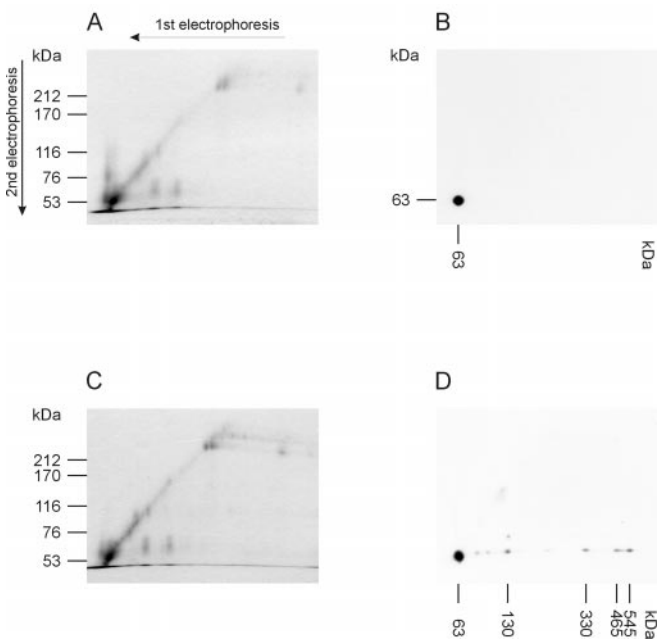

FIG. 5. **Cleavage and immunoblot analysis of BMDB cross-links of the channel α-subunit.** Washed ROS membranes were cross-linked with BMDB either in the absence of 8-Br-cGMP (*A* and *B*) or presence of 50 μM 8-Br-cGMP (*C* and *D*) and then subjected to electrophoresis in 5% β-mercaptoethanol on a 3.5–7.5% polyacrylamide gel. To oxidatively cleave the cross-links, the lanes were cut out, incubated in $NaIO_4$ as described (24), and analyzed on a second 3.5–7.5% SDS-polyacrylamide gel electrophoresis. Shown are Coomassie Blue-stained gels (*A* and *C*) and immunoblots labeled with the antibody, PMc 2G11, against the α-subunit of the channel (*B* and *D*). The directions of the first and second electrophoresis are indicated in *A*.

teins were cross-linked with BMDB in the presence of 8-Br-cGMP. As a control, non-NEM-treated membranes were cross-linked with BMDB as well, both in the absence and presence of 8-Br-cGMP (*lanes d* and *e*, respectively).

As expected, BMDB treatment of NEM-modified membranes did not yield any cross-linking bands of the β-subunit of the channel or of the exchanger; therefore, *lanes b* and *c* in *C* and *D* of Fig. 4 are indistinguishable from nontreated membranes (*lane a*), whereas cross-links were observed for membranes that had not been modified with NEM (Fig. 4, *lanes d* and *e*). Remarkably, the α-subunit of the channel could still be cross-linked with BMDB in the presence of 8-Br-cGMP if the membranes had been treated with NEM in the absence of 8-Br-cGMP (Fig. 4*B*, *lane b*) but not if they had been treated with NEM in the presence of 8-Br-cGMP (Fig. 4*B*, *lane c*). This finding indicates that 8-Br-cGMP binding leads to the exposure of at least one additional cysteine of the α-subunit but not of the β-subunit of the channel.

Cleavage of Cross-links and Two-dimensional Electrophoresis—The additional bands of the exchanger at 330, 465, and 545 kDa, which we observed in Western blots upon cross-linking with BMDB in the presence of 8-Br-cGMP, suggested that cross-links between channel and exchanger formed (see Fig. 3*D*, *lane d,* and Fig. 4*D*, *lane e*). However, no corresponding bands in one of the channel subunits were detected. Therefore, either the exchanger was cross-linked to some protein other than the channel or the affinity of the antibody against the channel subunit, which was covalently bound to the exchanger, was abolished after cross-linking. To discriminate between these two possibilities, the BMDB cross-links were cleaved in the polyacrylamide gel matrix, and the bands were analyzed by electrophoresis in a second polyacrylamide gel.

Fig. 5 shows the electrophoretic analysis of BMDB-treated ROS membranes after cleavage of the cross-link bridges. ROS membranes were cross-linked with BMDB either in the absence (*A* and *B*) or presence of 8-Br-cGMP (*C* and *D*) and electrophoresed on a SDS-polyacrylamide gel; the gel lanes were cut out, subjected to oxidative cleavage of the cross-linked proteins, and analyzed on a second polyacrylamide gel, as described (24). The blots were labeled with the antibody PMc 2G11 against the α-subunit of the channel. If the ROS membrane proteins had been cross-linked with BMDB in the absence of 8-Br-cGMP, the Western blot of this two-dimensional electrophoresis yielded only a single spot at 63 kDa for the α-subunit of the channel (Fig. 5*B*). This finding corroborates our conclusion (see above) that in the absence of 8-Br-cGMP, BMDB does not yield any cross-link of the α-subunit of the channel; this means in particular that also no cross-link formed between the α- and the β-subunit of the channel.

However, if cross-linking of ROS membranes was carried out in the presence of 8-Br-cGMP, there were several weak spots originating from the bands at 130, 330, 465, and 545 kDa of the first dimension electrophoresis gel in addition to the strong spot at 63 kDa (Fig. 5*D*). The 130-kDa spot indicated that the α-subunits of the channels were cross-linked to dimers. The positions of the latter three molecular masses were identical to the ones determined for the additional cross-linking bands of the exchanger (Fig. 3*D*, *lane d*) thus strongly suggesting that these cross-links were because of cross-links between the exchanger and the channel α-subunit. It should be noted that during oxidative cleavage of the cross-links in the one-dimensional gel, some protein was unavoidably lost, particularly proteins with lower molecular mass. For this reason, the spots originating from cross-links of the α-subunit were rather weak

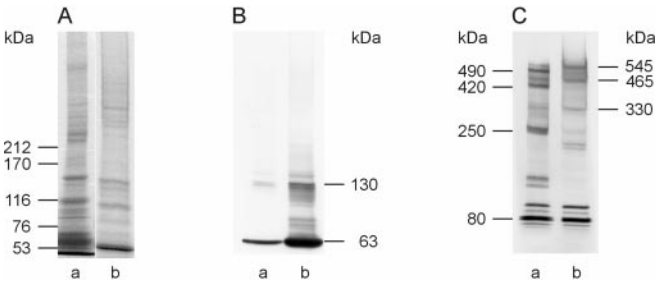

FIG. 6. **Purification of cGMP-gated channel after BMDB cross-linking of ROS membranes in the presence of 8-Br-cGMP.** To purify the cGMP-gated channel from ROS membranes that were cross-linked with BMDB in the presence of 50 μM 8-Br-cGMP, CaM affinity chromatography was carried out as described (see "Materials and Methods"). Solubilized proteins were analyzed by SDS-polyacrylamide gel electrophoresis before (*a,* 70 μg of protein) and after (*b,* 11 μg of protein) chromatography in a 3.5–7.5% gel. *A,* Coomassie Blue-stained gel; *B* and *C,* Western blot labeled consecutively with the antibody PMc 2G11 against the α-subunit of the channel (*B*) and the antibody PMe 1B3 against the exchanger (*C*).

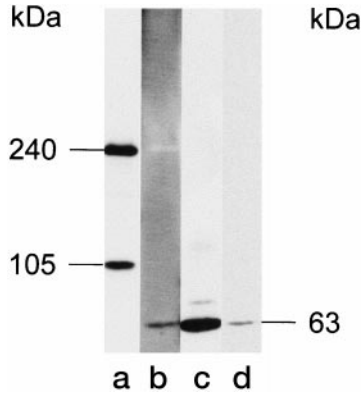

FIG. 7. **Western blot of purified cGMP-gated channel labeled with biotinylated purified exchanger.** The cGMP-gated channel was purified by CaM chromatography in 18 mM CHAPS (see "Materials and Methods"), subjected to SDS-polyacrylamide gel electrophoresis on a 3.5–7.5% polyacrylamide gel, blotted, and incubated overnight in phosphate-buffered saline at 4 °C. The blot was blocked for 30 min with 1% dry milk, incubated for 60 min in purified biotinylated exchanger (5 μg/ml), and consecutively labeled with antibodies against the β-subunit of the channel (PPc 32K, *lane a*), with the streptavidin horseradish peroxidase (*lane b*), with antibodies against the α-subunit of the channel (PMc 2G11, *lane c*), and the exchanger (PMe 1B3, *lane d*).

in Fig. 5*D*. This experiment has been repeated four times with similar results.

Finally, we infer that the immunoreactivity of the channel α-subunit against the monoclonal antibody PMc 2G11 was lost upon cross-linking of this subunit to the exchanger because the spots at 330, 465, and 545 kDa of the α-subunit were only detected after cleavage of the cross-links.

Purification of the Cross-linked Channel—A complex between the channel and the exchanger was further examined by purification of the channel after chemical cross-linking. The ROS membranes were first cross-linked with BMDB in the presence of 8-Br-cGMP and then solubilized in CHAPS, and the channel was purified by CaM affinity chromatography (28). Fig. 6 shows the solubilized ROS membrane proteins and the bound fraction eluted with 2 mM EDTA from the CaM column. A great amount of bound protein (11 μg) was applied on *lane b* of Fig. 6 to enable detection of the weak bands, too. It is apparent from the Western blots shown in Fig. 6 (*B* and *C*) that *lane b* not only contained channel protein but also a significant amount of exchanger. The exchanger protein was labeled mainly in the additional bands at 330, 465, and 545 kDa, which were obtained upon cross-linking of the membranes in the presence of 8-Br-cGMP (Fig. 6*C*, *lane b*). These bands were enriched, whereas the proteins of the strong bands of *lane a* at 250, 420, and 490 kDa were eluted in the nonbound fraction (not shown) and, therefore, almost completely absent in *lane b*. Moreover, although the α-subunit, the 130-kDa cross-link, and several other cross-linking bands between 63 and 150 kDa of the channel were labeled, none of the cGMP-dependent cross-linking bands of the exchanger were detected with the antibody PMc 2G11 against the α-subunit of the channel, again supporting our inference that the monoclonal antibody PMc 2G11 against the α-subunit does not recognize bands because of the cross-links between the α-subunit and the exchanger.

Protein Overlay Assay—In the following, we examined whether there is a direct interaction between the α-subunit of the channel and the exchanger. The cGMP-gated channel was purified by CaM affinity chromatography, and the subunits were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a protein-immobilizing membrane. This blot was incubated with purified exchanger that had been biotinylated at the thiol groups. If probed with streptavidin horseradish peroxidase, this blot exhibited a distinct band at the location of the α-subunit of the channel (Fig. 7, *lane b*) indicating a binding affinity of the exchanger to the α-subunit. The background was due to unspecific binding of the exchanger to the membrane; interestingly, there was significantly less background staining at the location of the β-subunit of the channel indicating that the exchanger did not bind to this subunit. As a control experiment, a second blot of the purified channel was prepared that had not been incubated in biotinylated exchanger; after streptavidin horseradish peroxidase treatment of this blot, no labeling could be detected in the enhanced chemiluminescence assay (not shown).

The bound streptavidin (but not the exchanger) of the blot shown in Fig. 7, *lane b,* could be completely washed off under denaturing conditions, so that no labeling was detected anymore in the enhanced chemiluminescence assay (not shown). This blot was reprobed repeatedly with antibodies against the α-subunit (*lane c*) and the β-subunit (*lane a*) of the channel and with antibodies against the exchanger (*lane d*). Similar to *lane b*, *lane d* of Fig. 7 showed a distinct band at 63 kDa indicating again the binding of exchanger to the α-subunit of the channel. These findings were fully reproducible in five experiments. Taken together, these results demonstrate that the exchanger binds to the α-subunit but not to the β-subunit of the channel.

DISCUSSION

Previously, we reported Ca^{2+} flux experiments that suggested that the cGMP-gated channel and Na/Ca-K exchanger were associated in the plasma membrane of bovine photoreceptors (1). This hypothesis is now strongly corroborated by the following biochemical findings reported in this study: (*a*) if purification of the channel by CaM affinity chromatography was carried out in 10 mM CHAPS, a significant fraction of exchanger was co-purified, which could not be fully removed by a second CaM affinity chromatography step; (*b*) channel and exchanger could be partially cross-linked with dimaleimides; (*c*) the cross-links between channel and exchanger were specific because these bands were only observed if cross-linking was performed in the presence of 8-Br-cGMP; and (*d*) when the subunits of the purified channel were separated by SDS-polyacrylamide gel electrophoresis and blotted, the exchanger bound preferentially to the α-subunit but not to the β-subunit of the channel.

For the following reasons, thiol-specific cross-linking of channel and exchanger was likely to be specific. Both exchanger and channel constitute only a minor protein fraction of the plasma membrane of photoreceptors, the major protein being rhodopsin; on a molar basis, rhodopsin exceeds channel and exchanger by more than a factor of ten. Moreover, each rhodopsin molecule contains 10 cysteines, whereas the Na/Ca-K exchanger contains only 6 cysteines, the α-subunit of the channel contains 6 cysteines, and the β-subunit contains 15 cysteines. Therefore, if unspecific cross-linking occurred, one should expect to obtain also cross-links between rhodopsin and channel or cross-links between rhodopsin and exchanger. However, Western blots of cross-linked and purified exchanger and channel were not labeled with the monoclonal antibody, Rho 1D4, against rhodopsin (not shown).

Thiol-specific cross-linking in the presence of 8-Br-cGMP resulted in three additional bands because of cross-links between channel and exchanger and in a cross-linking band, which indicate a dimer of the channel α-subunit. The cross-linking bands of the exchanger at 330, 465, and 545 kDa indicate cross-links of one α-subunit either to the exchanger monomer or to the cross-link between the 150-kDa exchanger fragment with the exchanger or to the exchanger dimer, respectively. Both, the multitude of possible cross-links and the reaction kinetics may be responsible for the low cross-linking efficiency.

Although the cGMP-gated channel is composed of α- and β-subunits we did not observe a cross-link between these two channel subunits by thiol-specific cross-linking. Instead, the β-subunit yielded a huge number of cross-links, which were not because of covalent bonds of the β-subunit with the α-subunit or with the exchanger. We did not analyze these cross-links further; we infer that channel and exchanger were only part of a still larger supramolecular assembly. This presumption is strongly supported by a very recent study showing that in rod photoreceptors, glutamic acid-rich proteins interact with components of the cGMP-signaling pathway (33).

Remarkably, the channel and exchanger retained some binding affinity even after solubilization in CHAPS, which depended on the detergent concentration. Interestingly, a similar dependence on the CHAPS concentration has been reported for the channel activity upon reconstitution in lipid vesicles (34). An indication of an interaction of channel and exchanger was also reported recently (35). Moreover, even after SDS denaturation and electrophoretic separation of the channel subunits, the exchanger bound clearly to the α-subunit but not to the β-subunit. The latter result fits nicely with the finding that the α-subunit but not the β-subunit of the channel could be cross-linked with the exchanger.

Recently, we reported that in photoreceptor plasma membranes, the Na/Ca-K exchanger could be cross-linked as a dimer, but there was no indication for dimerization of the exchanger molecules upon solubilization in CHAPS or Triton X-100 (24). Here, we show that (*a*) the exchanger bound to the α-subunit of the channel and (*b*) the α-subunits formed a dimer in the channel. Moreover, even after solubilization the binding affinity of the exchanger to the α-subunit of the channel was evident. These findings suggest that the close proximity of two exchanger molecules in the plasma membrane enabling thiol-specific linking to a homodimer was probably because of binding of two exchanger molecules to the α-subunit dimer of the channel rather than to a significant binding affinity between two exchanger molecules themselves. This consideration implies that all exchanger molecules were bound to the channel because the exchanger could be almost completely cross-linked by disulfide bridges (24) suggesting a molar ratio of exchanger to channel of 2:1.

This inference is in general agreement with previous studies in which this ratio was reported to be about 1.5:1 (36) and 2:1 (1). The cGMP-gated channel was suggested to have a tetrameric structure (37, 38) with the CaM binding site being localized on the β-subunit (39, 40). Moreover, CaM binding was found to be slightly cooperative with a Hill coefficient of 1.3 (41) indicating that there are two β-subunits/channel. Taken together, the complex of exchanger and channel appears to have the structure $x_2\alpha_2\beta_2$, if x denotes one exchanger molecule, and $\alpha_2\beta_2$, the subunit assembly of one channel molecule.

The results reported here strongly support the hypothesis inferred from the previous study (1) that the cGMP-gated channel and exchanger are associated in rod outer segment plasma membranes. The close proximity of source and sink of the cytosolic Ca^{2+} in rod outer segments suggests that Ca^{2+} signaling may constitute a local process in photoreceptors. However, it should be stressed that Ca^{2+} influx into rod outer segment is a permanent process in the nonexcited photoreceptor. If the Ca^{2+} ions entering the rod outer segment should be kept in a microdomain near the channel, one should expect that (*a*) the kinetic parameters governing Ca^{2+} diffusion are consistent with such a mechanism and (*b*) the target molecules for the Ca^{2+} signaling pathway are close to the channel as well. The latter expectation is supported by a recent report (33). Further studies are required to examine if Ca^{2+} signaling in photoreceptors is in fact a local process.

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