

Subunit 2 (or β) of retinal rod cGMP-gated cation channel is a component of the 240-kDa channel-associated protein and mediates Ca^{2+} -calmodulin modulation

T.-Y. CHEN*, M. ILLING†, L. L. MOLDAY†, Y.-T. HSU†, K.-W. YAU*‡§¶, AND R. S. MOLDAY†¶

Departments of *Neuroscience and †Ophthalmology and ‡Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and §Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

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ABSTRACT The cGMP-gated cation channel mediating visual transduction in retinal rods was recently found to comprise at least two subunits, 1 and 2 (or α and β). SDS gels of the purified channel show, in addition to a 63-kDa protein band (subunit 1), a 240-kDa protein band that binds Ca^{2+} -calmodulin, a modulator of the channel. To examine any connection between subunit 2 and the 240-kDa protein, cGMP-gated channels formed from the expressed cloned subunits in human embryonic kidney (HEK) 293 cells were tested for Ca^{2+} -calmodulin effect. Homooligomeric channels formed by subunit 1 alone showed no sensitivity to Ca^{2+} -calmodulin, and neither did heterooligomeric channels formed by subunit 1 and the short alternatively spliced form of subunit 2 (2a). By contrast, the cGMP half-activation constant ($K_{1/2}$) for heterooligomeric channels formed from subunit 1 and the long form of subunit 2 (2b) was increased 1.5- to 2-fold by Ca^{2+} -calmodulin, similar to the increase observed for the native channel. In Western blots of rod outer segment membranes, a subunit 2-specific antibody also recognized the 240-kDa protein. Finally, amino acid sequences derived from peptide fragments of the bovine 240-kDa protein showed $\approx 80\%$ identity to regions of subunit 2b of the human channel. These results together suggest that subunit 2b of the rod channel is a component of the 240-kDa protein and that it mediates the Ca^{2+} -calmodulin modulation of the channel.

Vertebrate rod photoreceptors respond to light with a membrane hyperpolarization, produced by a G protein-linked enzyme cascade that leads to cGMP hydrolysis and the consequent closure of a cGMP-gated cation channel open in darkness (1–4). This ion channel has been extensively studied both electrophysiologically and biochemically (1, 5, 6). Furthermore, a 63-kDa protein capable of forming such cGMP-gated channels has been purified from rod outer segments and molecularly cloned (7–9). In an SDS gel of the purified native channel, two protein bands are prominent: the 63-kDa protein and a 240-kDa protein (7, 10, 11). Recently, Ca^{2+} -calmodulin has also been shown to lower the apparent affinity of the native channel for cGMP (12). In the same study, gel-overlay experiments indicated that Ca^{2+} -calmodulin binds to the 240-kDa protein but not the 63-kDa protein; presumably, this binding allosterically leads to the change in affinity of the channel for cGMP (12).

Most recently, the native rod cGMP-gated channel has been shown, contrary to previous belief, to be a heterooligomer composed of at least two subunit species (13). Subunit 1 (or α) is the 63-kDa protein (9, 14). Subunit 2 (or β) shows only about 30% overall amino acid identity to subunit 1. This second subunit is incapable of forming functional cGMP-gated channels by itself, but when coassembled with subunit

1 it reproduces several characteristics of the native channel, such as flickery channel openings and a high sensitivity to the blocker *L-cis*-diltiazem (13). Two alternatively spliced forms of subunit 2, named 2a and 2b, were identified, with their only difference being that 2b has a longer cytoplasmic N-terminal segment (13). The molecular mass based on amino acid sequence is ≈ 70 kDa for protein 2a and ≈ 100 kDa for protein 2b. Thus, the native rod cGMP-gated channel complex on an SDS gel is expected to give, in addition to the 63-kDa band corresponding to subunit 1, a protein band of perhaps 70 or 100 kDa corresponding to subunit 2. However, as pointed out above, the only prominent bands in such a gel are at 63 and 240 kDa (7, 11). The question therefore arises whether subunit 2 is part of the 240-kDa protein and is perhaps even the target for Ca^{2+} -calmodulin binding. To examine this question, we have expressed homooligomeric channels formed from subunit 1 and heterooligomeric channels formed from subunits 1 and 2 (a or b forms), all cloned from human retina, and tested them for Ca^{2+} -calmodulin sensitivity. At the same time, we have used a subunit 2-specific antibody to perform Western blotting of human rod outer segment (ROS) membranes, and we have also sequenced peptide fragments derived from the bovine 240-kDa protein. The overall results indeed confirm the presence of protein 2b in the 240-kDa protein and its involvement in Ca^{2+} -calmodulin modulation of the channel. Protein 2b is also likely to be the substrate for calmodulin binding, though this remains to be confirmed.

MATERIALS AND METHODS

Vectors and cDNAs. The cDNAs encoding the human rod cGMP channel subunit 1 (hRCNC1; see refs. 13 and 14) and subunits 2a and 2b (hRCNC2a and 2b; see ref. 13) were inserted into a pCIS expression vector (Genentech) containing a cytomegalovirus (CMV) promoter. The construct RSV-Tag contains the simian virus 40 tumor (T) antigen under the control of the Rous sarcoma virus (RSV) promoter. See refs. 13–15.

Transient Transfection of Human Embryonic Kidney (HEK) 293 Cells. Ten micrograms of expression vector DNA was mixed with 10 μg of Bluescript (Stratagene) carrier DNA and 0.5–1.0 μg of RSV-Tag in 250 mM CaCl_2 in a final volume of 250 μl ; for cotransfections, cDNAs for the two subunits (10 μg each) were added together. The mixture was then added dropwise to 250 μl of 50 mM NaHepes, pH 7.1/280 mM NaCl/2.8 mM Na_2HPO_4 . The solution containing the precipitate was next added to 20–30% confluent HEK 293 cells growing in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After incubation in an atmosphere containing 5% CO_2 for 5–6 hr, the medium was aspirated and the cells were washed in phosphate-buffered saline before fresh medium was added again. Incubation was then continued for 2–3

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Abbreviation: ROS, rod outer segment.

¶To whom reprint requests should be addressed.

days, at which time electrophysiological recording or Western blotting was performed. For negative controls in the Western blotting, cells were also transfected as above, using pCIS vector without insert.

Western Blotting of Expressed Channels. HEK 293 cells transfected with cDNAs were harvested in lysis buffer containing 50 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, leupeptin at 0.1 mg/ml, and 1 mM phenylmethanesulfonyl fluoride. The cells were broken with a probe sonicator for 30 sec and the membrane fraction was pelleted with a benchtop ultracentrifuge. The membrane pellet was then resuspended in 300 μ l of lysis buffer; typically 10–30 μ l of the protein sample was loaded into a lane in the SDS gel. After electrophoresis the proteins in the gel were transferred onto nitrocellulose paper (Schleicher & Schuell), and the blot was then incubated with the primary antibody (mouse monoclonal antibody PMc 1D1, specific for subunit 1, and rabbit polyclonal antibody JH486, specific for subunit 2) overnight. The primary antibodies were labeled with 1:5000-diluted horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibodies and detected by the ECL chemiluminescence system (Amersham).

Electrophysiological Recording of the Expressed Channels. Electrical recordings from inside-out membrane patches excised from transfected HEK 293 cells were made by using an Axopatch 1D patch-clamp amplifier at bandwidth dc to 1 kHz (13–15). The signals were either recorded on a tape recorder or digitized directly into a computer for measuring current amplitude. The recording pipettes were fabricated from borosilicate glass and had tip lumen diameters of about 1 μ m. Seal resistances of the excised membrane patches were typically 2–10 G Ω . The pipette contained 140 mM NaCl, 5 mM KCl, 1 mM NaEGTA, and 10 mM NaHepes at pH 7.4 (0-Ca²⁺ solution). The bath contained either 0-Ca²⁺ solution or 50 μ M free Ca²⁺ solution, which has the same composition as 0-Ca²⁺ solution except having 704 μ M CaCl₂ and 2 mM sodium nitrilotriacetate instead of NaEGTA to give the correct buffered Ca²⁺ concentration. Appropriate concentrations of cGMP were added to the bath solution. To compare the cGMP dose–response relations in the presence and absence of calmodulin, the relation was first measured in 0-Ca²⁺ bath solution; the membrane patch was then perfused with a solution containing 50 μ M free Ca²⁺ and 250 nM calmodulin for 1 min before the dose–response relation was measured again in the Ca²⁺–calmodulin solution.

Western Blotting of Human ROS Membrane Proteins and the Immunoprecipitated Channel. Human ROSs were prepared under normal light by sucrose gradient centrifugation, and ROS membranes were isolated by hypotonic lysis of ROSs as previously described (16). For immunoprecipitation experiments, 1 mg of ROS membranes, solubilized in 0.5 ml of 1% Triton X-100/0.01 M Tris-HCl, pH 7.4/0.15 M KCl, was incubated at 4°C for 30 min with 0.1 ml of PMc 1D1 antibody-Sepharose 2B (11, 17). The unbound fraction was collected and the beads were washed with 20 ml of wash buffer consisting of 0.01 M Tris-HCl at pH 7.4, 0.15 M KCl, and 0.1% Triton X-100. The bound fraction was eluted from the beads with 0.1 ml of wash buffer containing 4% SDS. ROS membranes and the unbound and bound fractions were electrophoresed on SDS/8% polyacrylamide gels for detection of the channel subunits by Western blotting as described for the expressed channels.

Microsequencing of Peptide Fragments from Bovine 240-kDa Protein. The peptides giving amino acid sequences 2–5 (see Fig. 4) were generated as follows. The bovine rod channel was isolated on a calmodulin-Sepharose column (12). Typically, 80 mg of ROS membranes in solubilization buffer (20 mM Tris-HCl, pH 7.4/150 mM NaCl/1 mM dithiothreitol/2 mM CaCl₂/15 mM CHAPS or 1% Triton X-100) was applied to a 1 \times 5 cm calmodulin-Sepharose column at 4°C.

The column was washed with 10 column volumes of solubilization buffer, and then fragments were eluted with the same buffer except that 2 mM CaCl₂ was replaced with 2 mM EDTA. The 240-kDa protein was separated from the 63-kDa channel subunit by SDS/polyacrylamide gel electrophoresis and cleaved with CNBr and/or Endoproteinase Lys-C (Boehringer Mannheim) (18). Peptides were isolated by reverse-phase HPLC for N-terminal sequence analysis. The peptide giving sequence 1 in Fig. 4 was generated by digestion of bovine ROS membranes with porcine pancreatic kallikrein (0.25 unit/mg of ROS protein; 30 min at 25°C) and isolated on a calmodulin-Sepharose column.

RESULTS

Ca²⁺–Calmodulin Modulation of Expressed Human Rod Channels in HEK 293 Cells. Three varieties of human rod channels were expressed in HEK 293 cells and tested for sensitivity to Ca²⁺–calmodulin by using excised-patch recording: homooligomeric channels formed by subunit 1 alone (hRCNC1), heterooligomeric channels formed by subunit 1 and subunit 2a (hRCNC1 + hRCNC2a), and heterooligomeric channels formed by subunit 1 and subunit 2b (hRCNC1 + hRCNC2b). In Fig. 1 *Upper* the membrane current in each trace was induced by 70 μ M cGMP in three bath-solution conditions: 0 Ca²⁺, 50 μ M Ca²⁺, and 50 μ M Ca²⁺–250 nM calmodulin. For all three kinds of channels, 50 μ M Ca²⁺ alone had very little effect on the cGMP-induced current. In the presence of 50 μ M Ca²⁺–250 nM calmodulin, the current was unaffected for the channel formed by hRCNC1 alone or by hRCNC1 + hRCNC2a, but it was decreased for the channel formed by hRCNC1 + hRCNC2b. This current reduction did not reverse rapidly after removal of calmodulin, and did so only when Ca²⁺ was also removed, suggesting that the Ca²⁺–calmodulin complex bound tightly to the channel. In Fig. 1 *Lower* the complete experimental results for each kind of channel are shown in the form of averaged dose–response relations. It is clear that Ca²⁺–calmodulin affects neither the apparent cGMP affinity nor the maximum current for the hRCNC1 and the hRCNC1 + hRCNC2a channels. For the hRCNC1 + hRCNC2b channels, however, Ca²⁺–calmodulin increases the $K_{1/2}$ of the cGMP dose–response curve by about 70% (from 78 μ M in 0 Ca²⁺ to 134 μ M in 50 μ M Ca²⁺–250 nM calmodulin). This degree of $K_{1/2}$ increase is similar to that observed previously in native rod membrane (ref. 12; see also refs. 19 and 20). These results suggest that subunit 2b mediates the Ca²⁺–calmodulin sensitivity of the native rod channel.

Western Blotting of Expressed Human Rod Channels and of Human ROS Membranes. To find out whether subunit 2b is indeed a component of the 240-kDa protein, we determined whether an antibody against the human rod channel subunit 2 recognized the 240-kDa protein band in Western blots of human ROS membranes and of the immunoprecipitated channel complex. First, we determined the apparent molecular masses of the expressed human subunits and the specificity of the antibodies. Total membrane proteins from HEK 293 cells transfected with the corresponding cDNAs or the pCIS vector alone were run on an SDS gel for Western blotting with monoclonal antibodies PMc 1D1, directed against the C-terminal region of the bovine rod channel subunit 1 (21, 22), and JH486, a polyclonal antibody directed against the C terminus of the human rod channel subunit 2 (ref. 13; see also Fig. 4). As shown in Fig. 2, the PMc 1D1 antibody also stained the human homolog of the rod channel subunit 1—i.e., hRCNC1 (see also ref. 22). The expressed hRCNC1 protein migrated to a position closely corresponding to the predicted molecular mass of 80 kDa. This value is higher than the 63 kDa observed for the native protein in ROS membranes (refs. 21 and 22; see also Fig. 3 here), a difference

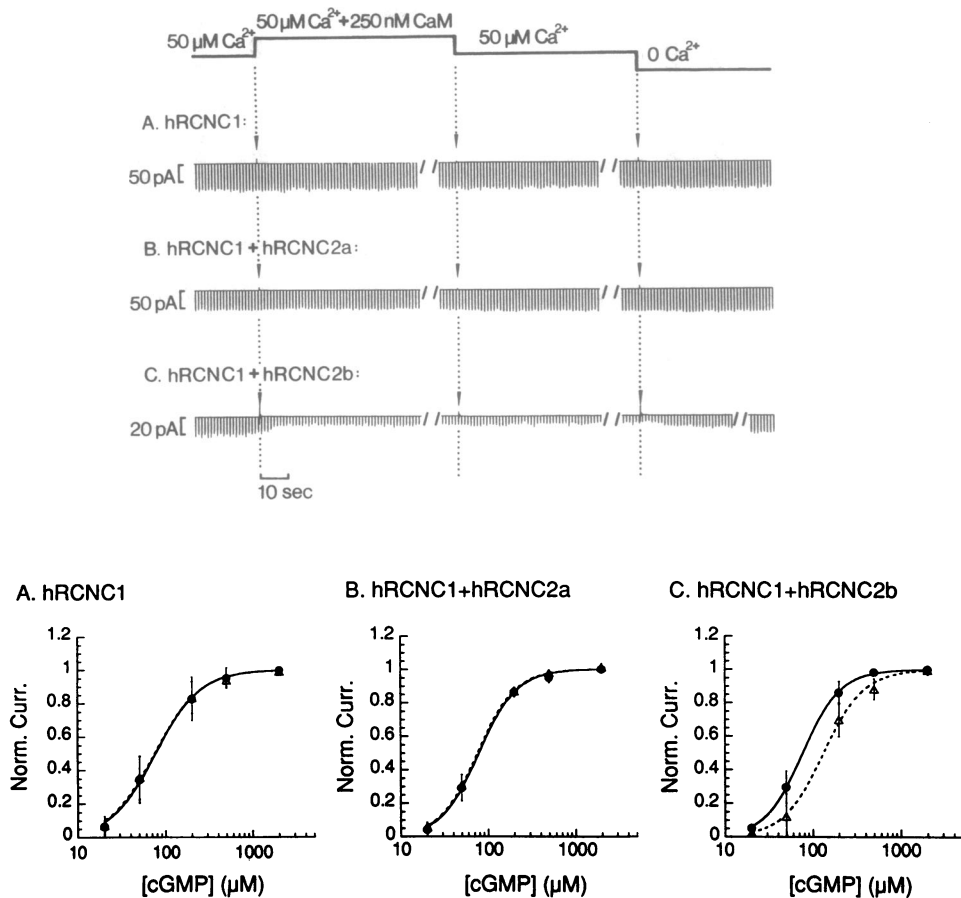


FIG. 1. Effect of Ca^{2+} -calmodulin on expressed human rod channels. (*Upper*) Each trace shows electrical recordings from a membrane patch excised from an HEK 293 cell transfected with the indicated cDNA. cGMP was $70 \mu\text{M}$ throughout. Membrane potential was maintained at 0 mV and a 60-msec voltage pulse to -60 mV was given once per second to monitor the cGMP-induced current. Leak currents in all three traces have been removed. Note that Ca^{2+} -calmodulin affects only the current through heterooligomeric channels formed by coexpression of hRCNC1 and hRCNC2b. (*Lower*) Averaged cGMP dose-response relations for the various expressed rod channels in 0 Ca^{2+} (\bullet - \bullet) and in $50 \mu\text{M Ca}^{2+}$ -250 nM calmodulin (Δ - Δ) conditions. The cGMP-induced currents were measured at -60 mV and normalized with respect to the current amplitude at 2 mM cGMP in 0-Ca^{2+} solution. The curves fitted to the data are drawn according to the Hill equation, $I_{\text{norm}} = C^n / [C^n + K_{1/2}^n]$, where I_{norm} is the normalized current, C is the cGMP concentration, and n is the Hill coefficient. The fitted $K_{1/2}$ and Hill coefficient in 0 Ca^{2+} and $50 \mu\text{M Ca}^{2+}$ -250 nM calmodulin are, respectively, as follows: (A) $76.1 \mu\text{M}$, 1.7; $74.3 \mu\text{M}$, 1.7 (average of four patches). (B) $79.3 \mu\text{M}$, 2.0; $76.2 \mu\text{M}$, 2.0 (average of four patches). (C) $77.8 \mu\text{M}$, 2.0; $134.1 \mu\text{M}$, 1.9 (average of six patches).

attributed in bovine rods to the removal of the first 92 amino acids by post-translational cleavage (22). Antibody JH486 stained both hRCNC2a and hRCNC2b, as expected. On the SDS gel, the expressed hRCNC2a has an apparent molecular mass close to the predicted 70 kDa. Subunit hRCNC2b, on the other hand, ran mainly as a doublet at 130–150 kDa. The reason for the doublet is unclear, but the higher apparent molecular mass compared with the predicted 102 kDa is probably due to the high content of glutamic residues at its N terminus (13). In addition, the expressed hRCNC2b shows a band of still higher molecular mass that may represent dimer formation. The Western blot of HEK 293 cells transfected with the pCIS vector alone did not show any staining, confirming the specificity of the JH486 and PMc 1D1 antibodies.

Next, we looked for the presence of subunit 2 in the 240-kDa protein of the native channel. Human ROS membranes were prepared, and the rod cGMP-gated channel was immunoprecipitated with the PMc 1D1 antibody coupled to Sepharose 2B. Three protein samples were run on an SDS gel for Western blotting: total ROS membrane proteins, the fraction bound to PMc 1D1-Sepharose, and the unbound fraction. As shown in Fig. 3, the JH486 antibody specifically stained a 240-kDa band in both total ROS membranes and the PMc 1D1-immunoprecipitated fraction. Another monoclonal

antibody, PMc 2G11, which is against the bovine rod channel subunit 1 (22), recognized the 63-kDa human homolog in both ROS membranes and the immunoprecipitated channel.

The above results suggest that the 240-kDa protein indeed contains the cGMP-gated channel subunit 2. Since the 240-kDa band binds Ca^{2+} -calmodulin (12), a modulator shown by the experiment of Fig. 1 to act selectively through protein 2b, it seems quite likely that at least protein 2b must be part of the 240-kDa protein. On the other hand, the apparent molecular mass of protein 2b is considerably lower than 240 kDa, suggesting the presence of another component in the latter protein.

Microsequencing of Peptide Fragments from Bovine 240-kDa Protein. Conclusive evidence for the presence of subunit 2b in the 240-kDa protein came from microsequencing of the latter. Five peptide fragments were obtained by digesting the 240-kDa protein from bovine ROS membrane with CNBr, CNBr/Endoproteinase Lys-C, or kallikrein. Most of these peptides were purified by HPLC, but a 102-kDa fragment was purified by Ca^{2+} -calmodulin affinity chromatography. These peptide fragments were subjected to microsequencing, and the amino acid sequences thus obtained were aligned with the amino acid sequence derived from the cDNA clone of the human rod channel subunit 2b (13). There is 77% identity between these sequences and 2b in an overall span of about

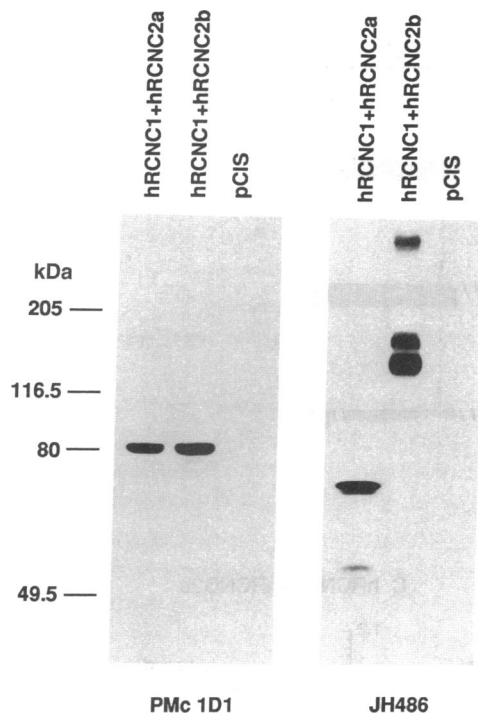


FIG. 2. Western blots of expressed human rod channels labeled with subunit-specific antibodies, PMc 1D1 (*Left*) and JH486 (*Right*). HEK 293 cells were transfected with the indicated combinations of cDNAs or pCIS vector alone. Cells were harvested 3 days after transfection, and roughly equal amounts of total membrane protein from each dish were loaded into 9% polyacrylamide gels. After transferring onto nitrocellulose membranes, the blots were labeled with the two antibodies.

86 amino acids, with the remainder being mostly conservative changes and reflecting probably just species differences (Fig. 4). Three peptide sequences (labeled 1–3 in Fig. 4) can be aligned to the N-terminal region of protein 2b, which is not present in protein 2a. Since sequence 1 was derived from the 102-kDa peptide fragment purified by Ca^{2+} -calmodulin affinity chromatography described above, this suggests that protein 2b probably binds Ca^{2+} -calmodulin.

In addition to the above five peptide fragments, other fragments with amino acid sequences completely different from the sequence of protein 2b have been obtained from digests of the 240-kDa protein (23), confirming that the 240-kDa protein contains an additional component as suggested in the previous section.

DISCUSSION

The 240-kDa protein in retinal ROSs identified in cGMP-gated channel purification studies was found to be tightly associated with the channel-forming 63-kDa protein, and it was originally suggested to be a cytoskeleton-like protein because of its immunochemical cross-reactivity with spectrin (10, 11). In the present study, however, we have found that subunit 2 or β of the channel actually is part of the 240-kDa protein. This finding explains the puzzling observation by others that cGMP binds not only to the 63-kDa protein but also to the 240-kDa protein (ref. 24; see also ref. 25). The reason is that subunit 2 has a cyclic nucleotide-binding site just as subunit 1 does (13).

Although protein 2b has a predicted molecular mass of 102 kDa, its apparent mass on SDS gels is between 130 and 150 kDa, presumably due to the high content of glutamic residues at its N terminus (13). Nonetheless, this value is still different from 240 kDa. The difference appears to be due to the

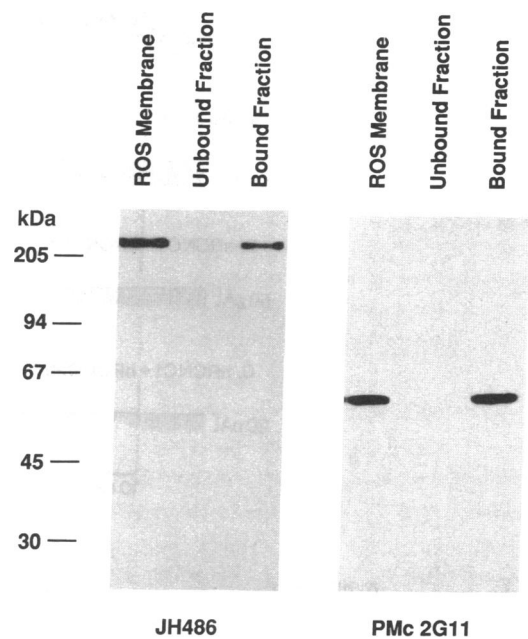


FIG. 3. Detection of rod channel subunit 2 in human ROS membranes and in the immunoprecipitated cGMP-gated channel complex by Western blotting. The cGMP-gated channel was immunoprecipitated from human ROS membranes by using the anti-channel monoclonal antibody PMc 1D1 coupled to Sepharose 2B. Human ROS membranes, the fraction bound to PMc 1D1, and the unbound fraction were subjected to SDS gel electrophoresis and transferred to Immobilon-P membranes (Millipore) and labeled with the subunit 2-specific antibody JH486 or the subunit 1-specific antibody PMc 2G11.

presence of another peptide component of ≈ 65 -kDa predicted molecular mass in the 240-kDa protein (ref. 23; also M.I., C. A. Colville, A. J. Williams, and R.S.M., unpublished data). The molecular characterization of this component and its mode of attachment to protein 2b are still unclear. Since the two proteins do not separate even in the presence of SDS, there is the intriguing possibility that they actually make up one single large protein. Protein 2b and the 65-kDa peptide fragment may represent alternatively spliced products of a single gene, with a full-length transcript giving rise to the 240-kDa protein.

A combination of protein 2b and the 65-kDa component may account for the overall 240 kDa. Protein 2a is also unlikely to be present as a separate subunit of the native rod channel, because the antibody JH486 labeled no additional band besides the 240-kDa band in the Western blot of the purified channel. Finally, it is worth noting that previous immunocytochemical staining has suggested that protein 2a, instead of protein 2b, is present in the ROS (13). This conclusion was arrived at from the observation that, while the antibody JH486 stains ROSs in a frozen retinal section, another polyclonal antibody, JH487, raised against an epitope on the extra N-terminal segment of 2b absent in 2a, fails to stain the ROS. In the present study, we have reexamined this point with Western blots and also found that, while JH487 clearly stains the expressed protein 2b, it labels only faintly the 240-kDa protein in Western blots of human ROS membranes (data not shown). These findings may suggest that the epitope for the antibody JH487 in the 240-kDa protein is for some reason not well exposed.

Since the presence of protein 2b, but not 2a, is required for the Ca^{2+} -calmodulin modulation, this suggests that the extra N-terminal segment on 2b must be important for this modulation. The molecular mechanism underlying the modulation, however, remains to be examined. The evidence presented in

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MPRELSRIIEEKEDEEEEEEEEEEEVTEVLVLLDSCVVSQVGVGQSEEDGTRPQSTSD -60
QKLWEEVGEAEAKKAEKAEKAEAEVAAEEAEKPEQDAETKKEPEAEAAEASSGVPAIK -120
QHPEVQVEDTDADSCPLMAEENPPSTVLPFPFPAKSDTLIVPSSASGTRKRKLPSEDEEA -180
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
IAEENPPSPVQLPLSPAKSDTLAVFGSATGSLR RLFSQ
          ①          ②          ③
EELKALSPAESPVVAWSDPTTKDQDRRAASTASTNSAIINDRLQELVKLFKERTKV -240
KEKLLDPDVTSDSESPKPSPAKKAPEPAPDTKPAEAEFVEEEHYCDMLCCKFKHRPWKKY -300
*
QFPQSIDPLTNLMYVLWLFVVMWANNWCNLI FVRWAFPHYQTFDNIHHWLLMDYLCDIY -360
FLDITVFQTRLQFVRGGDIITDKDKMRNNYLKSRFRKMDLLSLPLDFLYLKVGVNPLLR -420
LPRCLKYMAFFEFNSRLESILSKAYVYRVIRTAYLLYSLHLNSCLYTWASAYGGLGSTH -480
WVYDGVGNSYIRCIYFAVKTLITIGGLPDKTLFEIVFQLLNLYFTGVFAFSVMIGQMRDV -540
VGAATAGQTYRSCMDSTVKYMNFKYKIPKSVQNKVKTWYEYTWHSQMLDESELMVQLPD -600
KMRLDLAIDVNYNIVSKVALFQCGDQRQMFDMKRLRSVVYLFDNDYVCKKGEIGREMYII -660
QAGQVQLGGPDGKSVLVTLKAGSVFGEISLLAVGGGNRRRTANVVAHGFTNLFILDKKDL -720
NEILVHYEPESQKLLRRKKARRMRLRNNPKKEKSVLILPPRAGTPKLFNAALAMTGRMGKK -780
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
LRNNKPKK-KSVLILPPRA GAK
          ④
GAKGGKLAHLRARLKELAAEAQAQQLVEQAQSSQVKGEEGSAAPDQHTHPKEAATD -840
: : : : : : : : : : : : : : : : : : : : : : : :
GGRRGLALLRARLKELAAEAAR
          ⑤
FPAPRTPEPPGSPSPSPASLGRPEGEEGFAPEEHSVRCMSPGPEPGEQILSVKM -900
PEEREKAE -909
JH486

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FIG. 4. Alignment of peptide sequences from the bovine 240-kDa protein with the protein sequence of subunit 2 of the human cGMP-gated channel (hRCNC2). Peptides were purified by HPLC from CNBr digests (sequences 4 and 5) or CNBr/Endoproteinase Lys-C digests (sequences 2 and 3) of the bovine 240-kDa channel protein for N-terminal sequence analysis. Sequence 1 was obtained from a 105-kDa peptide isolated by calmodulin-affinity purification of kalikrein-digested bovine ROS membranes. The sequence of the human rod channel subunit 2 is from Chen *et al.* (13). The asterisk indicates the beginning methionine of protein 2a. The region marked JH486 indicates the peptide sequence that this antibody was raised against.

Results strongly suggests that 2b is actually the substrate for calmodulin binding, but this conclusion remains tentative until the relevant calmodulin-binding site is identified. Recently, a Ca^{2+} -calmodulin modulation has also been observed for the cyclic nucleotide-gated channel mediating olfactory transduction (26), though in this case the modulation appears to be through subunit 1 of the channel. An apparently direct modulation by Ca^{2+} -calmodulin has been reported previously for several ion channels, though the underlying molecular mechanisms are so far unclear (27). With a wealth of information about their structure and function, the cyclic nucleotide-gated channels may be useful for studying the calmodulin modulation more closely.

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