Disulfide Bonds in the Extracellular Calcium-Polyvalent Cation-sensing Receptor Correlate with Dimer Formation and Its Response to Divalent Cations in Vitro

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Extracellular calcium/polyvalent cation-sensing receptors (CaR) couple to G proteins and contain highly conserved extracellular cysteine residues. Immunoblotting of proteins from rat kidney inner medullary collecting duct endosomes with CaR-specific antibodies reveals alterations in the apparent molecular mass of CaR depending on protein denaturation conditions. When denatured by SDS under nonreducing conditions, CaR migrates as a putative dimeric species of 240–310 kDa. This is twice the predicted molecular mass of the CaR monomer observed after SDS denaturation in the presence of sulfhydryl-reducing agents. In sucrose density gradients, Triton X-100-solubilized CaR sediments as a 220-kDa complex, not explainable by binding of G proteins to CaR monomers. Treatment of Triton-soluble CaR with divalent (Ca2+, Mg2+) and trivalent (Gd3+) metal ion CaR agonists, but not monovalent ions (Na+, K+), partially shifts the electrophoretic mobility of CaR under denaturing conditions from a predominantly monomeric to a putative dimeric species on immunoblots in a manner similar to their rank order of functional potency for CaR activation (Gd3+ > Ca2+ > Mg2+). This Ca2+ effect is blocked by pretreatment with N-ethylmaleimide. We conclude that disulfide bonds present in CaRs mediate formation of dimers that are preserved in Triton X-100 solution. In addition, CaR exposure to Ca2+ induces formation of additional disulfide bonds within the Triton-soluble CaR complex.

The binding of divalent (Ca2+, Mg2+), trivalent (Gd3+), and polyvalent (neomycin, protamine) cations to the extracellular domain of CaRs1 initiates a variety of signal transduction cascades via G protein coupling (1–3). Following their expression in both oocytes (4, 5) and cultured human embryonic kidney (HEK) cells (6–8), CaRs have been characterized pharmacologically. Immunoblotting of these exogenously expressed CaRs reveals multiple CaR-specific bands (7–9). To account for these diverse CaR species, it has been suggested that CaRs undergo post-translational modification including glycosylation (8). Moreover, studies using the same antibodies to probe endogenous CaRs present in rat kidney epithelial cells from both the thick ascending limb of Henle (10) as well as from IMCD (11) have revealed multiple CaR species exhibiting similar molecular masses to those reported for exogenously expressed CaRs. However, at present no detailed studies have examined the origin of these multiple CaR species that are present on immunoblots. In this report, we have utilized a combination of immunoblotting, SDS gel permeation chromatography, and sucrose density gradient centrifugation of Triton-solubilized CaR prepared from rat kidney IMCD to characterize CaR associations in both nonionic and ionic detergents. The CaR examined in this study is present in purified endosomes that are derived exclusively from the apical membrane of IMCD epithelial cells (11, 12). Utilization of an endogenous CaR present in a defined intracellular compartment rather than a recombinant CaR species expressed in cultured cells at high levels and in multiple intracellular compartments precludes a potential complication of studying multiple CaR species that are actually present in different compartments within cells. The data reported here provide evidence that a major form of CaR is a putative dimeric species that is present following solubilization with both Triton X-100 and SDS detergents and perhaps in the cell membrane itself.

Previous studies have demonstrated that the non-glycosylated CaR polypeptide migrates as a band of approximately 120 kDa on SDS-PAGE immunoblots in close agreement with the putative molecular mass predicted from its corresponding cDNA (13). The presence of glycosylated CaRs possessing an estimated molecular mass of 120–200 kDa has been demonstrated by conversion of this larger 120–200-kDa CaR band to a 120-kDa band after digestion with glycosidases (8, 13). However, a number of studies (7–11) have reported the presence of multiple CaR-reactive protein bands greater than 200 kDa on SDS-PAGE immunoblots that are not substantially effected by glycosidase digestion. These data suggest that CaRs may also associate with, or be bound covalently to, other protein(s) including a second CaR molecule. Support for the possibility of a dimeric CaR species is suggested by recent data showing that the structurally related metabotropic glutamate receptor, mGlur5, is a disulfide-linked homodimer in the plasma membrane of cells that exhibits a high molecular weight band on SDS-PAGE immunoblots (14). Putative mGlur5 dimer formation appears to be mediated by sulfhydryl (SH) linkages present in the N-terminal region of the mGlur5 extracellular domain that possesses at least 3 Cys residues that are identical in all CaRs as well as other mGlur5s (13, 15–21).

1 The abbreviations used are: CaR, Extracellular calcium/polyvalent cation-sensing receptors; HEK, human embryonic kidney; IMCD, inner medullary collecting duct; PAGE, polyacrylamide gel electrophoresis; SH, sulfhydryl; Nem, N-ethylmaleimide; DTT, dithiothreitol; bME, β-mercaptoprotoehanol; GTPγS, guanosine 5′-3-O-(thio)triphosphate; mGlur, metabotropic glutamate receptor.

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**EXPERIMENTAL PROCEDURES**

**Materials**

Male Sprague-Dawley rats (200–250 g) were purchased from Charles River Laboratories (Cambridge, MA). Various items were obtained from the following sources: EuCl₃ (Molecular Probes Inc., Eugene, OR); Triton X-100 (Bio-Rad); acrylamide (Amersham Pharmacia Biotech); polyvinylidenefluoride membrane (MSI, Westbrook, MA); ECL reagents (Amersham Pharmacia Biotech); and autoradiography film (NEN Life Science Products). All other chemicals were purchased from Sigma. Anti-CaR mouse monoclonal antibody, raised to amino acids 214–235 of the extracellular domain of the human parathyroid CaR (15), was obtained from NPS Pharmaceuticals (Salt Lake City, UT), and rabbit polyclonal anti-CaR antibody (A4641), raised to amino acids 215–237 of bovine parathyroid CaR, was a gift of Dr. Steven Hebert (Division of Nephrology, Vanderbilt University School of Medicine, Nashville, TN). Other antisera utilized included rabbit anti-Band III antibody, a gift of Dr. Samuel E. Lux (Harvard Medical School, Boston, MA), and rabbit anti-Gα₁₅, a gift of A. Tashjian (Harvard University, Boston, MA), and rabbit anti-Gα₁₃, and anti-Gα₁₁, antibodies (Upstate Bio-technology Inc., Lake Placid, NY).

**Methods**

**Isolation and Triton X-100 Solubilization of Endosomes**—After animals were sacrificed by cervical dislocation under anesthesia (100 mg/kg pentobarbital, intraperitoneally), endosomes were prepared as described previously (12) and utilized either immediately or stored at −80 °C with identical results. Endosomal protein content was determined by the method of Bradford (22). Endosomal proteins (100–200 μg) were solubilized by incubation on ice for 30 min in solubilization buffer: 8.3 mM Tris (pH 7.4), 125 mM NaCl, 1.25 mM pepstatin, 4 μM leupeptin, 4.8 μM phenylmethylsulfonyl fluoride, and 1.0% (v/v) Triton X-100 (final concentrations). The resulting mixture was then centrifuged at 100,000× g for 30 min to remove Triton X-100-insoluble material, and the supernatant containing Triton-soluble endosomal proteins was collected and aliquoted into equal volumes for the experiments detailed below. To prepare crude membranes, inner medullary papilla homogenate was spun at 2,500 × g for 5 min, and the resulting postnuclear supernatant was spun at 100,000 × g (30 min) to give a crude inner medullary pellet.

**Sucrose Density Gradient Ultracentrifugation**—Triton-X-100-soluble endosomal proteins were layered on top of a 5–20% (v/v) sucrose density gradient (8 ml) in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.05% (v/v) Triton X-100 and ultracentrifuged in an SW41 rotor at 39,000 rpm for 8 h at 4 °C. Individual fractions were then collected from the bottom of the tube and processed for Western blotting. The mass of Triton-X-100-soluble CaR was estimated by comparison of its mobility with those of standard proteins including bovine liver catalase, Band III, and hemoglobin as described by Martin and Ames (23) and Clarke (24).

**SDS Gel Permeation Chromatography**—Samples of SDS-denatured endosomal proteins were applied to a 1.3 × 45-cm Sephacryl 4-B (Amersham Pharmacia Biotech) column equilibrated with 50 mM Tris (pH 8), 0.1% SDS buffer in the absence or presence of 10 mM DTT. One-mL fractions were collected for analyses of both protein amount (A₂₈₀₅₆₉) and content (Western blotting as described below). A single column was utilized for multiple chromatographic runs.

**Western Blot Analysis of CaR Proteins**—Five-fold concentrated Laemmli buffer (0.32 M Tris (pH 6.8), 5% (v/v) sodium dodecyl sulfate, 25% (v/v) glycerol, 1% (v/v) bromphenol blue) was added in a 1:4 ratio to sample proteins that were incubated in the presence or absence of various SH group reducing agents at various temperatures and then fractionated on SDS-polyacrylamide gels (22). Proteins were then transferred electrophoretically to polyvinylidene fluoride membrane in blotting buffer (25 mM Tris, 200 mM glycine, 15% (v/v) methanol) containing 0.5% (v/v) SDS (8) so as to improve the transfer of the proteins >100 kDa (26, 27). The membrane was then incubated in 1% (v/v) bovine serum albumin (20 min) to block nonspecific binding sites, followed by a 1-h incubation in either anti-CaR mouse monoclonal antibody (214–237 of the extracellular domain) or anti-CaR rabbit antisera (125–235). After washing to remove all nonspecifically bound anti-CaR antibodies, blots were then exposed to either horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000 for 1 h. All incubations and intervening washes were performed in Tween/TBS solution (15 mM Tris (pH 8), 150 mM NaCl, 0.1% (v/v) Tween 20) at room temperature, and blots were developed with the ECL kit as detailed in the manufacturer’s instructions. Immunoreactivity was quantified by densitometry and is expressed as percent of total CaR immunoreactivity in the lane (±S.E.), and statistical significance was determined by paired t test.

**RESULTS**

**Specific Anti-CaR Monoclonal and Polyclonal Antibodies Both Identify Multiple CaR Bands in Immunoblots from the Inner Medulla of Rat Kidney**—Fig. 1 shows how various experimental manipulations affect the distribution of CaR-reactive bands in immunoblots after fractionation of IMCD endosomal proteins by SDS-PAGE. As shown in panel a, a CaR-specific mouse monoclonal antibody identifies sequence determinants in a highly acidic region of the extracellular domain present in all CaRs reported to date (9). When purified endosomes are solubilized directly in SDS-PAGE Laemmli buffer in the absence of SH-reducing agents (Fig. 1, panel a, lane 1), greater than 90% of the CaR immunoreactivity is present in a broad band of approximate molecular mass 240–310 kDa. In contrast, addition of β-mercaptoethanol (βME) to a final concentration of 143 mM results in both a diminution in the intensity of the broad 240–310-kDa band as well as the appearance of CaR monomer species. Solubilization of an identical amount of endosomal protein with 1% (v/v) Triton X-100 yielded a Triton X-100-insoluble pellet (Tx-Ins; lane 3) and Triton X-100-soluble supernatant (Tx-Sol; lane 4) that both contained multiple CaR-reactive species following SDS denaturation with 143 mM βME. Panel B, Coomassie Blue-stained SDS-polyacrylamide gel showing the protein content of the samples described in panel a. The results shown are representative of a minimum of three separate experiments. Panel C, Western blot showing total endosome proteins solubilized in the absence (lane 1) or presence (lane 2) of 143 mM βME and immunoblotted in a manner identical to that shown in panel a, except using a rabbit polyclonal anti-CaR antisemur (n = 2). Arrowheads on the left indicate the apparent molecular masses of CaR-reactive species observed.
purified subcellular fraction. Following solubilization in the nonionic detergent Triton X-100, integral membrane proteins such as Band III (28) and bacteriorhodopsin (29) maintain their nonionic detergent Triton X-100, integral membrane proteins such as Band III (28) and bacteriorhodopsin (29) maintain their non-ionic nature in the absence of reducing agents. Following solubilization in Laemmli buffer containing SDS and 143 mM βME, denatured and Triton X-100-solubilized endosomal proteins were subjected to denaturation in Laemmli buffer containing 143 mM βME where the protein mixture was applied to the gel after a 30-min incubation at 20 °C (lanes 1 and 2) or 100 °C (lanes 3 and 4). Panel B, immunoblot of Triton X-100-solubilized endosomal proteins denatured in Laemmli buffer in either the absence of a reducing agent (lanes 1 and 2) or containing either 143 mM βME (lanes 4 and 5), 10 mM DTTP (lane 5), 109 mM β-mercaptoethanol (βME, lane 6) or 1 mM NEM (lane 7). Immunoblotting of lanes 1 and 2 was performed using anti-CaR monoclonal antibody preincubated with a 100-fold molar excess of immunizing peptide. These immunoblots are each representative of a minimum of three separate experiments. Panel C, immunoblot of total endosomal proteins after preincubation of native endosomes on ice for 5 min in the absence (lanes 1 and 2) or presence of 1 mM NEM (lane 3) or 1 mM EDTA. 1 mM EDTA (lane 4) prior to their solubilization in Laemmli buffer containing SDS and 143 mM βME (lane 1) or SDS alone (lanes 2–4). Panel D, kidneys collected from anesthetized rats perfused with phosphate-buffered saline in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 mM NEM were homogenized in buffer in the continued absence or presence of NEM (1 mM). Crude IMCD membranes were then prepared and solubilized in Laemmli buffer under nonreducing (lanes 1 and 3) or reducing (lanes 2 and 4) conditions. Panels C and D are each representative of two independent experiments. Arrowheads on the left indicate the apparent molecular masses of the CaR-reactive species observed.

**Fig. 2.** Effects of temperature and SH group modification on CaR immunoreactivity. Panel A, immunoblot of total (Tot, lanes 1 and 3) and Triton X-100-solubilized (Txsol, lanes 2 and 4) endosomes subjected to denaturation in Laemmli buffer containing 143 mM βME where the protein mixture was applied to the gel after a 30-min incubation at either 20 °C (lanes 1 and 2) or 100 °C (lanes 3 and 4). Panel B, immunoblot of Triton X-100-solubilized endosomal proteins denatured in Laemmli buffer in either the absence of a reducing agent (lanes 1 and 2) or containing either 143 mM βME (lanes 4 and 5), 10 mM DTTP (lane 5), 109 mM β-mercaptoethanol (βME, lane 6) or 1 mM NEM (lane 7). Immunoblotting of lanes 1 and 2 was performed using anti-CaR monoclonal antibody preincubated with a 100-fold molar excess of immunizing peptide. These immunoblots are each representative of a minimum of three separate experiments. Panel C, immunoblot of total endosomal proteins after preincubation of native endosomes on ice for 5 min in the absence (lanes 1 and 2) or presence of 1 mM NEM (lane 3) or 1 mM EDTA. 1 mM EDTA (lane 4) prior to their solubilization in Laemmli buffer containing SDS and 143 mM βME (lane 1) or SDS alone (lanes 2–4). Panel D, kidneys collected from anesthetized rats perfused with phosphate-buffered saline in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 mM NEM were homogenized in buffer in the continued absence or presence of NEM (1 mM). Crude IMCD membranes were then prepared and solubilized in Laemmli buffer under nonreducing (lanes 1 and 3) or reducing (lanes 2 and 4) conditions. Panels C and D are each representative of two independent experiments. Arrowheads on the left indicate the apparent molecular masses of the CaR-reactive species observed.
individual fractions were then collected from the bottom of the gradient, solubilized in SDS-Laemmli buffer containing 10 mM DTT, and assayed for CaR content by quantitative immunoblotting. The apparent molecular mass of Triton X-100-solubilized CaR was estimated from a standard curve constructed from the mobilities of bovine liver catalase (230 kDa), Band III (175 kDa), and hemoglobin (64 kDa), which were included as native protein markers in the same sucrose gradient. Portions of the CaR immunoblots are presented to show the actual CaR-reactive species present in the gradient fractions: Panel A, i, and Panel B, ii, represent the fractions that contained maximal CaR immunoreactivity for each sample, and Panel A, iii, shows the glycosylated and non-glycosylated CaR content of each fraction across the sucrose gradient CaR band. The experiment was performed 5 times, and in each case a second identical tube and sample (○–○) was centrifuged, as described above, in the presence of either 100 μM GTPγS (panel A, n = 2) or 10 mM DTT (panel B, n = 3).

**Triton X-100-solubilized CaR Exists as a Complex of Approximately 220 kDa**—Triton X-100-soluble extracts of endosomal proteins were subjected to ultracentrifugation in sucrose density gradients containing Triton X-100 in the absence of sulfhydryl reducing agents and the sedimentation of CaR compared with that of marker proteins of known native molecular weights. As shown in Fig. 3, panel A, i, CaR immunoreactivity was observed in a single discrete region of the sucrose gradient in close proximity to the catalase (230 kDa) marker, where the peak CaR immunoreactivity corresponded to an estimated molecular mass of approximately 220 kDa (Figs. 1 and 2). As shown in panel A, ii and iii, of Fig. 3, SDS-PAGE analysis of each respective sucrose gradient fraction revealed that each displayed a series of CaR bands including 121, 138–169, or 240–310 kDa identical in appearance to those shown in Figs. 1 and 2. Note that the dissociated monomeric CaR species are not equally represented in each gradient fraction (Fig. 3, panel A, iii). For example, the 121-kDa CaR protein is localized only in fractions 12–14, whereas the 138–169-kDa CaR protein is enriched in fractions 10–13.

To determine whether the molecular mass of the detergent-solubilized CaR complex could be accounted for by the presence of a bound G protein as described for the galanin (32), kainate (33), melatonin (34), opioid (35), and pancreastatin (36) receptors, a second identical sample was centrifuged in the presence of 10 mM GTPγS (Fig. 3, panel A, i). SDS-PAGE analysis of each respective sucrose gradient fraction revealed that each displayed a series of CaR bands including 138–169, 121, 240–310 kDa identical in appearance to those shown in Figs. 1 and 2. Note that the dissociated monomeric CaR species are not equally represented in each gradient fraction (Fig. 3, panel A, iii). For example, the 121-kDa CaR protein is localized only in fractions 12–14, whereas the 138–169-kDa CaR protein is enriched in fractions 10–13.

**S-S bonds responsible for the 240–310-kDa CaR complex preexist** prior to tissue processing.

**Divalent and Trivalent Cations Alters Its Electrophoretic Mobility in SDS-PAGE**—Denaturation and SH-reducing agents under standard conditions utilized to isolate most membrane preparations. Derivation of CaR SH groups with NEM to prevent SH oxidation and the formation of disulfide bonds prior to or during SDS denaturation fail to replicate the dissociation of the CaR complex achieved by SH-reducing agents such as β-mercaptoethanol or DTT. These data suggest that reduction of pre-existing S-S bonds are primarily responsible for the formation of CaR monomers on immunoblots. However, a minimum of 40% of the CaR immunoreactive protein contained in purified apical membrane endosomes is successfully reduced to CaR monomers, whereas the remainder is resistant to treatment with multiple SH-reducing agents.

**Exposure of Triton X-100-solubilized CaR to Divalent and Trivalent Cations Alters Its Electrophoretic Mobility in SDS-PAGE**—Denaturation and SH-reducing agents under standard conditions utilized to isolate most membrane preparations. Derivation of CaR SH groups with NEM to prevent SH oxidation and the formation of disulfide bonds prior to or during SDS denaturation fail to replicate the dissociation of the CaR complex achieved by SH-reducing agents such as β-mercaptoethanol or DTT. These data suggest that reduction of pre-existing S-S bonds are primarily responsible for the formation of CaR monomers on immunoblots. However, a minimum of 40% of the CaR immunoreactive protein contained in purified apical membrane endosomes is successfully reduced to CaR monomers, whereas the remainder is resistant to treatment with multiple SH-reducing agents.

**SDS-solubilized CaR Is Decreased in Size upon Reduction**—To validate further the data shown in Figs. 1–3, SDS gel permeation chromatography was utilized to demonstrate that DTT alters the apparent size of SDS-denatured CaR. As shown in Fig. 4, fractionation of SDS-solubilized endosomes by gel permeation chromatography shows that the $K_v$ of SDS-solubilized CaR without DTT (0.41) is smaller as compared with a paired identical sample denatured and chromatographed in the presence of 10 mM DTT ($K_v$ 0.5). These data are in agreement with SDS-PAGE immunoblotting analyses and suggest that SH-reducing agents decrease the molecular mass of SDS-denatured CaR from a value consistent with a CaR dimeric species to that consistent with a monomeric species.

The simplest interpretation of data displayed in Figs. 1–4 is that CaR protein in rat IMCD endosomes is present as a dimeric species that can only be partially dissociated by SDS denaturation and SH-reducing agents under standard conditions utilized to isolate most membrane preparations. Derivation of CaR SH groups with NEM to prevent SH oxidation and the formation of disulfide bonds prior to or during SDS denaturation fail to replicate the dissociation of the CaR complex achieved by SH-reducing agents such as β-mercaptoethanol or DTT. These data suggest that reduction of pre-existing S-S bonds are primarily responsible for the formation of CaR monomers on immunoblots. However, a minimum of 40% of the CaR immunoreactive protein contained in purified apical membrane endosomes is successfully reduced to CaR monomers, whereas the remainder is resistant to treatment with multiple SH-reducing agents.
CaR Is a Disulfide-linked Dimer

Calcium-induced Alteration of CaR Electrophoretic Mobility Is Mediated by Disulfide Bonds within the CaR Complex—To determine whether exposure of CaR to Ca$^{2+}$ produces a structural alteration in CaR protein that prevents CaR monomer formation upon subsequent SDS denaturation, Triton X-100-solubilized CaR was incubated at either 4 °C (Fig. 6, lane 1) or 37 °C (Fig. 6, lane 3) prior to denaturation in SDS-Laemmli buffer containing 143 mM βME at 20 °C. As compared with 4 °C, CaR incubation at 37 °C consistently resulted in increased CaR immunoreactivity present in the 240–310-kDa range.

PAGE—Although the binding of divalent, trivalent, and polyvalent cations to CaRs are known to activate downstream signaling cascades (13, 37), only limited data exist investigating the structural alterations that occur in CaRs after ion binding. To examine whether the electrophoretic mobility of CaR is altered in a manner similar to the relative potencies of Ca$^{2+}$, Mg$^{2+}$, or Na$^+$ at 37 °C and then analyzed by SDS-PAGE immunoblotting as shown in Figs. 1–3. As shown in Fig. 5, Ca$^{2+}$ exposure of Triton X-100-solubilized CaR induced a concentration-dependent decrease in the intensity of the monomeric (121 and 138–169 kDa) CaR bands accompanied by an increase in the intensity of the 240–310-kDa species (20 ± 6% increase in 240–310-kDa signal induced by 20 mM Ca$^{2+}$, as percent of total CaR signal, p < 0.05, n = 4). A similar effect was also observed following Mg$^{2+}$ treatment (+16 ± 3% by 20 mM Mg$^{2+}$, p < 0.01, n = 4), but higher (20–40 mM) Mg$^{2+}$ concentrations were required to prevent formation of CaR monomers as compared with 10–20 mM Ca$^{2+}$ (Fig. 5). These effects appeared specific since exposure to 0–80 mM Na$^+$ produced no significant change in CaR monomer formation (+7 ± 6% by 40 mM Na$^+$, n = 3). These data shown in Fig. 5 are similar to the relative potencies of Ca$^{2+}$ and Mg$^{2+}$ obtained from analyses of recombinant CaRs. In a similar manner, exposure to trivalent lanthanides that also activate CaRs, including Gd$^{3+}$ (200 μM) and Eu$^{3+}$ (200 μM), also prevented formation of monomeric CaR species on SDS-PAGE immunoblots (Fig. 5). It should be noted, however, that the appearance of the high molecular weight complex induced by lanthanides is different from that produced by Ca$^{2+}$ and Mg$^{2+}$, since the trailing edge of the lanthanide-induced complex is more diffuse and extends almost into the stacking gel. This is consistent with the possibility that Ca$^{2+}$ and Gd$^{3+}$ may actually activate the CaR at different binding sites (8, 38).

Fig. 4. Gel permeation chromatography of SDS-solubilized CaR under reducing and nonreducing conditions. A mixture of endosomes (600 μg of protein) and bovine serum albumin (1 mg) were solubilized in 2% SDS in either the absence or presence of 10 mM DTT and then fractionated through a Sephacryl 4B column in the absence (solid line) or presence (dashed line) of 10 mM DTT. The resulting fractions were mixed with Laemmli buffer in 143 mM βME and assayed for CaR content by quantitative immunoblotting (see “Experimental Procedures”). The elution coefficient ($K_{av}$) of denatured CaR was increased by the addition of DTT. In contrast, the $K_{av}$ of bovine serum albumin (as determined spectroscopically at 280 nm and by Coomassie Blue staining of an SDS-polyacrylamide gel) was constant in the presence and absence of DTT.

Fig. 5. Exposure of Triton X-100-solubilized CaR to divalent and trivalent cations but not NaCl alters CaR electrophoretic mobility. Triton X-100-solubilized CaR was incubated for 20 min at 37 °C in the presence of increasing concentrations of CaCl$_2$, MgCl$_2$, or NaCl or with either 200 μM GdCl$_3$, or 200 μM EuCl$_3$. The samples were then denatured in Laemmli buffer containing 143 mM βME at 20 °C and immunoblotted against monoclonal anti-CaR antibody (see “Experimental Procedures”). The NaCl concentrations given are in excess of the 125 mM NaCl present in every sample. These data shown are representative of a minimum of three separate identical experiments.

Fig. 6. The effect of temperature and Ca$^{2+}$ exposure on the distribution of Triton X-100-solubilized CaR immunoreactive bands. Triton X-100-solubilized CaR extracts were incubated for 10 min at either 4 or 37 °C in the presence or absence of 1 mM NEM. Subsequently, all samples were incubated for an additional 10 min in either the presence or absence of 20 mM Ca$^{2+}$. The resulting samples were all solubilized in Laemmli buffer containing 143 mM βME and immunoblotted as described under “Experimental Procedures.” The Western blot shown in A is representative of 3–6 separate experiments. The optical density of each 240–310-kDa CaR-reactive band was then quantified and is displayed histographically (B) as a percentage of the total CaR signal in the lane (mean ± S.E.) normalized to the 4 °C control. Statistical analysis is using the paired t test; **, p < 0.01 (lanes 3 versus 4); *, p < 0.05 (lanes 5 versus 6); ***, p < 0.001 (lanes 7 versus 8).
band with a corresponding decrease in monomer immunoreactivity of CaR. NEM pretreatment of the CaR samples exposed to 37 °C blocked the shift in CaR electrophoretic mobility to the larger 240–310-kDa band (Fig. 6, lane 3; p < 0.01, n = 6) but did not affect the distribution of CaR bands after incubation at 4 °C (Fig. 6, lane 2). These data suggest that a 10-min exposure of Triton X-100-solubilized CaR to 37 °C results in the formation of disulfide bonds that are not susceptible to reduction during subsequent SDS denaturation in the presence of SH-reducing agents. In a similar manner, exposure of Triton-soluble CaR to 20 mM Ca2+ at 4 °C produced a shift in CaR immunoreactivity identical to that produced by incubation at 37 °C that was also significantly inhibited by pre-exposure to 1 mM NEM (Fig. 6, lane 5; p < 0.05, n = 3). The shifts produced by either calcium or exposure to 37 °C are not additive, and their combination was also significantly attenuated by NEM pretreatment (Fig. 6, lane 8; p < 0.001, n = 6). These data suggest that exposure to Ca2+ induces an alteration in Triton X-100-solubilized CaR at 4 °C that is similar to that produced by exposure of CaR alone to 37 °C. Both treatments alter the ability of the combination of SDS and SH-reducing agents to fully denature CaRs to monomeric species.

**DISCUSSION**

These data reported here confirm and greatly extend earlier reports (8, 13) showing that the electrophoretic profile of CaR immunoreactive bands is more complex than simply nonglycosylated and glycosylated monomeric CaRs present on immunoblots prepared from CaR-containing protein mixtures. Whereas the presence of a high molecular weight CaR-immunoreactive band on immunoblots has been previously reported (2, 7–10), it has been unclear as to whether its origin represented a real biochemical association or was simply an artifact of denaturation in SDS-containing detergent solutions. Data presented in Figs. 1 and 2 show that SDS denaturation of rat kidney IMCD CaR in the absence of SH-reducing agents results in the formation of a single 240–310-kDa CaR band, as determined using both monoclonal and polyclonal anti-CaR antibodies. The specificity of this polyclonal anti-CaR antiserum has been demonstrated previously where no bands were observed in immunoblots with membranes prepared from mock-transfected HEK cells, whereas membranes from HEK cells transfected with human CaR and bovine parathyroid tissue possessed CaR bands identical to those reported here (8).

Addition of SH-reducing agents to SDS-denatured CaR reduces its apparent size as measured by gel permeation chromatography (Fig. 4) as well as diminishes the intensity of the 240–310-kDa band with a concomitant appearance of CaR-reactive bands of 121 and 138–169 kDa that correspond to non-glycosylated and glycosylated monomeric CaRs, respectively (Figs. 1 and 2). Perfusion of intact kidney tissue with NEM to prevent any SH oxidation followed by immediate processing of membranes in NEM fails to prevent isolation of a CaR dimeric species. However, denaturation of CaR in SDS in the presence of SH-reducing agents is capable of only partial conversion of the total CaR immunoreactivity to CaR monomeric species. As shown in Figs. 1 and 2, a significant proportion (0–60%) of immunoreactive CaR protein remains as a discrete 240–310-kDa band. At present, the exact nature of this large SDS-resistant CaR is unknown. However, data shown in Fig. 2 suggest that this SDS-resistant CaR complex may possess S-S linkages that resist the combination of SDS and reducing agents, and its formation is increased by both exposure to elevated temperature (Fig. 2, panel A; Fig. 5) and prolonged exposure of membrane preparations to buffers that do not contain SH-reducing agents (Fig. 2, panel D). These data do not permit us to distinguish whether intramolecular or intermolecular S-S bonds contribute to formation of this SDS-resistant CaR complex.

To study the interactions of CaR with itself or other proteins, CaR was solubilized in the nonionic detergent Triton X-100 and analyzed by a combination of sucrose gradient sedimentation and SDS-PAGE immunoblotting. As shown in panel A of Fig. 1, 58 ± 5% of Triton X-100-solubilized CaR was converted to CaR monomers upon denaturation in SDS-reducing agents, whereas the Triton X-100-insoluble CaR fraction was correspondingly enriched for the larger 240–310-kDa CaR species. As shown in Fig. 3, Triton X-100-soluble CaR sediments as a complex of approximately 220 kDa in the presence or absence of SH-reducing agents as determined in sucrose gradients. Previous analyses of Triton X-100-solubilized membrane proteins such as Band 3, the major anion exchanger of the human red cell, using identical sucrose gradient sedimentation techniques have demonstrated that Triton X-100 solubilization does not disrupt intermolecular protein-protein associations between Band 3 dimers or interfere with the binding of other cytoskeletal and cytoplasmic proteins to Band 3 (24). These data dis-
played in Fig. 3 show the presence of a 220-kDa high molecular weight immunoreactivity on CaR immunoblots (9). Taken to
gether, these data reported here and previous reports (14) suggest that an ability to form dimeric CaR species may be a
conserved between all of the mGluRs (19–21) and all CaRs so
far reported (13, 15–18). Indeed, Table I shows that the con-
servative G protein-coupled receptors (33–37, 41) since its sedimentation is not altered by preincubation with GTPγS (Fig. 3, panel A).
Instead, the IMCD CaR appears to correspond more closely to
the muscarinic acetylcholine (42), neurotensin (43), and gastrin-releasing peptide (44) receptors that do not possess asso-
ciated G proteins after nonionic detergent solubilization.

As described under “Results,” careful analyses of the mono-
meric CaR species shown in Fig. 3, panel A, iii, reveal that the Triton X-100-soluble CaR complex present in individual frac-
tions of the sucrose gradient contains a nonrandom mixture of
glycosylated and non-glycosylated CaR monomers. The sim-
plest interpretation of these data is that the Triton X-100-
solubilized CaR complex consists of CaR dimers composed of
the N terminus of the extracellular domain. Of note, this
mGluR5 region contains 4 Cys residues (19), 3 of which are
conserved between all of the mGluRs (19–21) and all CaRs so
far reported (13, 15–18). Indeed, Table I shows that the con-
servation of extracellular cysteine residues within the extra-
cellular domains of the various known members of this receptor
family is very high despite varying degrees of overall homology.
A previous report (45) using immunoblotting analyses of both
cells transfected with various mGluR species as well as specific
regions of rat brain shows the formation of specific bands of
approximately twice the apparent molecular mass of mono-
meric mGluRs after SDS denaturation in the presence of SH-
reducing agents. These data suggest that CaRs form dimers or
complexes via their extracellular domains. A truncated CaR
lacking all of the intracellular domain and 4 of the 7 trans-
membrane domains still exhibits dimer-like high molecular weight
immunoreactivity on CaR immunoblots (9). Taken to-
tgether, these data reported here and previous reports (14) suggest that an ability to form dimeric CaR species may be a
characteristic common to all members of this G protein-coupled receptor subfamily.

The data shown here suggest that both the IMCD CaR and
mGluR5 (14) are distinct from the receptor tyrosine kinases
that undergo dimerization principally upon agonist binding.
However, addition of Ca2+ to Triton X-100-solubilized CaR
significantly reduced the ability of SDS and SH-reducing agents to generate monomeric CaR species, raising the possi-
bility that Triton-soluble CaR may undergo structural modifi-
cation in response to metal ion agonist treatment. The effect of
Ca2+ was mimicked by other metal ion CaR agonists in a rank
order of potency equivalent to that determined in functional assays (Gd3+ >> Ca2+ >> Mg2+). These data, shown in Figs. 5
and 6, suggest that binding of divalent and valent cations to
the CaR molecule induces a conformational change in the CaR
complex that promotes oxidation of free sulfhydryl groups re-
sulting in a Ca2+-mediated reduction in CaR monomer forma-
tion upon subsequent exposure to SDS and SH-reducing agents. Although metal ions such as Ca2+ and Zn2+ can them-
selves promote oxidation of free sulfhydryl residues, the data
shown in Figs. 5 and 6 suggest these effects are not simply
onspecific, since Ca2+ and Mg2+ exposure causes a shift in
CaR electrophoretic mobility between discrete, monomeric, and
putative dimeric bands, as opposed to producing a broad smear
of CaR immunoreactivity along the lane which could indicate
random aggregation. Thus, the current data are most consist-
ent with the concept that putative dimeric CaR species become
stabilized upon addition of Ca2+ in vitro. Further work is
required to determine what proportion of CaRs actually exist
as either monomers or dimers in the membranes of intact cells
and whether an equilibrium between the two states has a
functional significance similar to that recently established for
the G protein-coupled δ opioid receptor (46).

The importance of extracellular domain disulfide bonds on
agonist-induced G protein-coupled receptor activation has been
investigated recently using site-directed mutagenesis studies of
both the thyrotropin-releasing hormone receptor (47, 48) and
gonadotropin-releasing hormone receptor (49). To date, most
studies of the CaR have investigated the expression of various
normal and mutant recombinant CaRs (obtained from natu-
really occurring mutations and by site-directed mutagenesis) in
oocytes (4, 5, 13) and HEK cells (6–9). The studies reported
here have suggested an important role for sulfhydryl groups in
formation of a putative CaR dimeric species and provide a
series of testable hypotheses that should be the object of future
studies of CaR using site-directed mutagenesis.

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