Interaction of the Ca\(^{2+}\)-sensing receptor with the inwardly rectifying potassium channels Kir4.1 and Kir4.2 results in inhibition of channel function

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1Department of Medicine and 2Department of Physiobiology, Case Western Reserve University, 3Louis Stokes Veterans Affairs Medical Center, and 4Rammelkamp Center for Research and Education, MetroHealth System Campus, Cleveland, Ohio; 5GI Diseases Research Unit, Hotel Dieu Hospital and Queen’s University, Kingston, Ontario, Canada; 6The Water and Salt Research Center, University of Aarhus, Aarhus, Denmark; and 7Department of Pharmacology II, Osaka University, Osaka, Japan

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Huang C, Sindic A, Hill CE, Hujer KM, Chan KW, Sassen M, Wu Z, Kurachi Y, Nielsen S, Romero MF, Miller RT. Interaction of the Ca\(^{2+}\)-sensing receptor with the inwardly rectifying potassium channels Kir4.1 and Kir4.2 results in inhibition of channel function. Am J Physiol Renal Physiol 292: F1073–F1081, 2007. First published November 22, 2006; doi:10.1152/ajprenal.00269.2006.—The Ca\(^{2+}\)-sensing receptor (CaR), a G protein-coupled receptor, is expressed in many epithelial tissues including the parathyroid glands, kidney, and GI tract. Although its role in regulating PTH levels and Ca\(^{2+}\) metabolism is best characterized, it may also regulate salt and water transport in the kidney as demonstrated by recent reports showing association of potent gain-of-function mutations in the CaR with a Bartter-like, salt-wasting phenotype. To determine whether this receptor interacts with novel proteins that control ion transport, we screened a human adult kidney cDNA library with the COOH-terminal 219 amino acid cytoplasmic tail of the CaR as bait using the yeast two-hybrid system. We identified two independent clones coding for the 125 aa from the COOH terminus of the inwardly rectifying potassium channel; HEK 293 cells; yeast two hybrid

THE CA\(^{2+}\)-SENSING RECEPTOR (CaR), a member of the G protein-coupled receptor (GPCR) superfamily, is expressed in the parathyroid glands, kidney, and other epithelial tissues. The primary physiological role of the CaR is to regulate PTH levels and Ca\(^{2+}\) balance, but it may also play an important role in the regulation of salt and water transport in the kidney (4, 14). Activation of the CaR mediates the acute adverse renal effects of hypercalcemia including reducing Na\(^{+}\), Cl\(^{-}\), K\(^{+}\), and H\(_{2}\)O reabsorption, the renal response to vasopressin, and aquaporin expression (4, 14, 29). Mutations of the human CaR gene cause disorders of mineral-ion homeostasis and lead to several human diseases. Loss-of-function mutations of the CaR cause familial hypocalciuric hypercalcemia (heterozygous) and neonatal severe hyperparathyroidism (homozygous) (29), and activating mutations cause autosomal dominant hypocalcemia (30). Recently, potent activating mutations of the CaR were identified as the cause of a Bartter-like syndrome characterized by volume depletion, Na\(^{+}\), Cl\(^{-}\), and K\(^{+}\) wasting, hypomagnesemia, hypercalciuria, and elevated rennin and aldosterone levels in addition to autosomal dominant hypocalcemia (40, 43).

Five genetically distinct abnormalities of transport proteins that lead to phenotypes characterized by salt-wasting (Bartter’s and Gitelman’s syndromes) have been described. Bartter’s syndrome can be caused by loss-of-function mutations of the Na\(^{+}-K\(^{-}\)-2Cl\(^{-}\) cotransporter (NKCC2), the renal outer medullary K\(^{+}\) channel (ROMK), the basolateral Cl\(^{-}\) channel (CIC-Kb) and a variant also characterized by sensorineural deafness is caused by loss of function mutations in an accessory protein for CIC-Kb, BSND, or barttin (2, 26, 38, 40). The hypocalciuric, hypomagnesemic variant of Bartter’s syndrome (Gitelman’s syndrome) is due to loss of function mutations of the thiazide-sensitive Na\(^{+}-\)Cl\(^{-}\) cotransporter (NCC) (26). The CaR represents a sixth gene, but with gain of function mutations, that can lead to a Bartter-like phenotype (40, 43).

Like a number of other receptors such as those for angiotensin (ANG) II, endothelin, and vasopressin (V1), the CaR couples to multiple G proteins (Go\(_{a}\), Go\(_{q}\), G\(_{\beta}y\), and Go\(_{12/13}\)) involved in distinct signaling pathways that can contribute to regulation of ion transport (14, 16, 17). Inhibition of adenyl cyclase due to CaR stimulation of Go\(_{a}\) with reduced cAMP accumulation inhibits the activity of NKCC2 and ROMK (22). Phospholipase A\(_{2}\) activated by the CaR via Go\(_{q}\) produces arachidonic acid metabolites that inhibit ROMK activity (10, 41). The activity of ROMK channels can also be regulated by phosphatidylinositol bisphosphate (PIP\(_{2}\)) hydrolysis and synthesis that are regulated by CaR-activated phospholipase C and phosphatidylinositol-4 kinase (16, 45). Although the CaR activates cellular signaling systems that can explain the inhibition
of NKCC2 and ROMK, other receptors such as those for ANG II, endothelin, and α-adrenergic agonists stimulate similar signaling pathways without causing Na⁺, Cl⁻, and K⁺ wasting to the same degree as the CaR. This difference in effects on salt transport between the CaR and other receptors suggests that the CaR may act via distinct mechanisms to regulate electrolyte metabolism such as direct interaction with channels, pumps, or transporters.

To identify additional proteins that interact with the CaR and that could explain its effects on electrolyte balance, we used the COOH-terminal 219 amino acids of the human CaR representing the cytoplasmic tail as bait to screen a human adult kidney cDNA library using the yeast two-hybrid assay. Using reciprocal immunoprecipitation and measurement of channel activity, we found that the CaR interacts with two inwardly rectifying potassium channels, Kir4.1 and Kir4.2, that are expressed in the distal nephron, and that the CaR inactivates these channels (19, 23). Regulation of this family of channels has not been studied extensively. Our results demonstrate that the CaR interacts directly with Kir4.1 and Kir4.2 and suggest a mechanism by which the CaR could inhibit electrolyte transport in the distal nephron.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Sigma or Fisher Scientific unless specified otherwise. The monoclonal anti-CaR and anti-HA antibodies and HEK 293 cells that stably express the wild-type CaR were described earlier (10, 11). G418 sulfate and cell culture reagents were purchased from Life Technologies. The SuperSignal West Pico chemiluminescent substrate and BCA protein assay reagent were obtained from Pierce. The polyclonal anti-Myc (A-14) and monoclonal anti-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-Kir4.1, and anti-ROMK antibodies, and their respective specific peptides were supplied by Alomone Laboratories (Jerusalem, Israel). The polyclonal anti-Kir4.1 antibody used for immunohistochemistry was obtained from Yoshihisa Kurachi, Osaka, Japan. The polyclonal anti-Kir4.2 antibody was provided by Dr. C.E. Hill, Queen’s University, Kingston, Ontario, Canada.

Yeast two-hybrid cloning. A cDNA representing the COOH-terminal 219 amino acids (aa) of the human CaR (CaR861–1078) in the bait plasmid pAS2.1 was used to screen a human adult kidney cDNA library in pACT2 (Invitrogen). The bait plasmid was transformed into S. cerevisiae strain AH109 by electroporation, mated with the pre-transformed library in S. cerevisiae strain Y187, and then plated on medium deficient in uracil, leucine, and histidine (37). The plates were replicated on media lacking histidine, leucine, and adenine. The colonies that grew under these conditions were tested for His+ and adenine. The colonies that grew under these conditions were tested for adenine. The colonies that grew under these conditions were tested for histidine. The colonies that grew under these conditions were tested for leucine.

Yeast two-hybrid cloning. A cDNA representing the COOH-terminal 219 amino acids (aa) of the human CaR (CaR861–1078) in the bait plasmid pAS2.1 was used to screen a human adult kidney cDNA library in pACT2 (Invitrogen). The bait plasmid was transformed into S. cerevisiae strain AH109 by electroporation, mated with the pre-transformed library in S. cerevisiae strain Y187, and then plated on medium deficient in uracil, leucine, and histidine (37). The plates were replicated on media lacking histidine, leucine, and adenine. The colonies that grew under these conditions were tested for His⁺ and adenine. The colonies that grew under these conditions were tested for adenine. The colonies that grew under these conditions were tested for histidine. The colonies that grew under these conditions were tested for leucine.

Expression of plasmids. To investigate the relationship between the Kir proteins (Kir4.2, Kir4.1, and Kir5.1) and the CaR without the limitation of specific antibodies, we made Myc- and HA-tagged constructs of each channel engineering epitope tags into the NH₂ terminus of the proteins. The cDNA coding for rat Kir4.2 (Kir4.2) was a generous gift from C. E. Hill (13). The Kir4.2pGem construct was digested with EcoRI and NotI and then ligated into EcoRI and NotI sites of pCruz-HA or pCruz-Myc vector. The cDNAs coding for rat Kir4.1 (Kir4.1) and rat Kir5.1 (Kir5.1) were generously provided by Stephen Tucker at the University Laboratory of Physiology, United Kingdom (28). The Kir4.1pBF construct was digested with EcoRI, blunt-ended, and then digested with KpnI. The Kir5.1pBF construct was digested with ApaI, blunt-ended, and then digested with KpnI. The pCruz-Myc or -HA constructs were digested with NotI, blunt-ended, and then digested with KpnI. The fragments were ligated into pCruz-Myc or pCruz-HA. All cDNA constructs were verified by direct sequencing. The ROMK pCDNA3.1 was a generous gift from Wenhui Wang at the New York Medical College, Valhalla, NY.

Transfection, immunoblotting, and immunoprecipitation. HEK-293 cells or HEK-293 cells that stably express the CaR were transiently transfected with the epitope-tagged channel constructs indicated (the Myc-tagged Kir4.1pCruz, the HA-tagged Kir4.2pCruz, the Myc-tagged Kir4.2pCruz, the HA-tagged Kir5.1pCruz, the Myc-tagged Kir5.1pCruz) or the ROMKpCDNA3 using the 55°C for 2 min for channel proteins or boiled for 5 min for the CaR and incubated at 37°C for 24–48 h. All cells were cultured in medium containing 1.8 mM CaCl₂. The medium was removed and washed once with 1× PBS. The cells were lysed with 1× RIPA buffer, and the lysates were centrifuged at 15,000 rpm for 1 h at 4°C. The protein concentrations in the cell lysates were determined using the BCA protein assay reagent with BSA as a standard and then adjusted to the same concentration with buffer. The samples were subjected to 11% SDS-PAGE, processed for immunoblotting, and the levels of protein expression were determined. The cellular lysates were used for coimmunoprecipitation as described (16). Briefly, either a monoclonal anti-CaR antibody or a polyclonal anti-Myc (A-14) antibody was separately loaded onto the Dynabead-protein A complex and slowly rotated for 2 h. The antibody-loaded Dynabead-protein A complex was rinsed twice, the beads were mixed with the various cell lysates, and rotated in the cold room overnight. The supernatants were discarded and the Dynabead-protein A complex was washed. Loading buffer was added and vortexed vigorously. The tubes were placed in Dynal-MPC to collect the sample buffer. The samples were heated at 55°C for 2 min for channel proteins or boiled for 5 min for the CaR and then subjected to SDS-PAGE for immunoblotting using the antibodies indicated. For coimmunoprecipitation studies using rat kidney membrane extracts, rat kidneys were isolated, and the cortex and medulla were separated. The cortex and medulla were then cut into small pieces and homogenized in a buffer containing 20 mM HEPES, pH 8.0, 2 mM MgCl₂, 1 mM EDTA, and protease inhibitors, and centrifuged at 2,000 rpm for 10 min. The supernatant was centrifuged at 13,500 rpm for 60 min to obtain crude membrane and cytosol fractions. The samples were used for immunoblotting to confirm the presence of the endogenous proteins. The crude membranes were extracted in 1× RIPA buffer for 60 min, centrifuged at 13,500 rpm for 60 min, the resulting supernatants were used for immunoprecipitation using an anti-CaR antibody, and the immuno precipitated samples were processed for immunoblotting using antibodies against the CaR, ROMK, Kir4.1, and Kir4.2 proteins.

Immunocytochemistry. The CaR- and Myc-Kir4.2-coexpressing HEK 293 cells were grown on glass coverslips for 2 days. At 40–60% confluence, the coverslips were fixed with cold 4% paraformaldehyde for 20 min and permeabilized with 0.05% saponin. The samples were processed for double immunofluorescence using our monoclonal anti-CaR antibody (16) and a polyclonal anti-Myc antibody (A-14) as the primary antibodies, and Alexa Fluor 488 goat anti-mouse IgG (green) and Alexa Fluor 594 goat anti-Rabbit IgG (red) as secondary antibodies. The coverslips were observed using fluorescence microscopy (Zeiss, Model LSM-5 Pascal) and images were collected using the Axiocam 200 program (Zeiss).

For immunoperoxidase staining, kidney blocks containing all kidney zones were dehydrated and embedded in paraffin.
embedded tissue was cut at 2 μm on a rotary microtome (Mircron). Staining was carried out using indirect immunoperoxidase. The sections were dewaxed and rehydrated, and endogenous peroxidase was blocked with 0.5% H2O2 in absolute methanol for 10 min at room temperature. To retrieve antigens, sections were treated with a 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EDTA and heated in a microwave oven for 10 min. Non specific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH4Cl for 30 min followed by PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with anti-Kir4.1 or anti-CaR antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. After being rinsed with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 3 x 10 min, the sections were incubated in horseradish peroxidase (HRP)- conjugated goat anti-rabbit immunoglobulin (DAKO P448) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. The samples were observed using a Leica DM RE light microscope.

**Electrophysiology.** Oocytes were harvested and prepared as previously described (36). Kir4.1 and Kir4.2 were subcloned into the oocyte expression vectors, pBF or pGEMSH respectively. Linearized samples were observed using a Leica DMRE light microscope.

**RESULTS**

Evidence for direct interaction of the CaR with the Kir4.2 channel. To identify proteins that interact with the CaR and that play important roles in the regulation of distal nephron function, we prepared a cDNA construct that corresponds to the COOH-terminal 219 aa (CaR860–1078) of the human CaR representing the intracellular cytoplasmic tail, to use as bait to screen a human adult kidney cDNA library. After plating on medium that lacked histidine, tryptophan, leucine, and adenine, we obtained 64 β-galactosidase-positive colonies. Two of the clones coded for an inwardly rectifying potassium channel and represented the COOH terminus of Kir4.2 (subfamily J, KCNJ15) from aa 243–375 and aa 254–375 (Fig. 1A). To identify the region(s) of the CaR COOH terminus that interacts with Kir4.2, we subcloned three fragments of the CaR COOH terminus (CaR860–908, CaR907–991, and CaR987–1078) into the pAS2.1 vector, mated them with one of the clones we isolated (Kir4.2243–375) in pACT2, and plated them on medium lacking histidine, tryptophan, leucine, and adenine. The Kir4.2 COOH terminus interacts with the CaR907–991 fragment (Fig. 1B), and the midregion of the CaR COOH terminus interacted with the COOH-terminal 125 amino acids of Kir4.2.

To determine whether the CaR and Kir4.2 interact in mammalian cells, we transiently coexpressed the CaR and Kir4.2 with a Myc epitope tag at its NH2 terminus in HEK-293 cells and carried out coimmunoprecipitations using our anti-CaR antibody. In Fig. 2A, the bands representing both the CaR and Kir4.2 were present in extracts of the cells where they were expressed, but not in the untransfected cells. Figure 2B shows that the specific bands representing Myc-Kir4.2 are coimmunoprecipitated with the CaR using our anti-CaR antibody as the immunoprecipitating antibody. This finding is consistent with our yeast two hybrid results.

**Interaction of the CaR and Kir4 is highly selective.** Kir4.2 and Kir4.1 are the only two known members of the Kir4 family and are both expressed in the distal nephron, potentially in the same region as the CaR (19, 23, 33). To determine whether the CaR interacts with Kir4.1, we transiently transfected Myc-Kir4.2 or Myc-Kir4.1 into HEK 293 cells that stably express the CaR, and the lysates were immunoprecipitated with either our anti-CaR antibody or a polyclonal anti-Myc antibody (A-14). In Fig. 3, left, the anti-CaR antibody was used for immunoprecipitation, and immunoprecipitation of the CaR was verified by immunoblotting the immunoprecipitate with the anti-CaR antibody (Fig. 3, left top). Coimmunoprecipitated Myc-Kir4.2 and Myc-Kir4.1 were identified by immunoblotting the same immunoprecipitate with an anti-Myc antibody. In the Fig. 3, bottom left, bands representing the Myc-tagged channel proteins are present only in lanes 3 and 4 where they
were coexpressed with the CaR but not in lanes where no immunoprecipitating antibody was used (lane 1) or where no channels were expressed (lane 2). The top band seen in all lanes is a nonspecific band. In Fig. 3, right, the polyclonal anti-Myc antibody (A-14) was used for immunoprecipitation. Bands representing the immunoprecipitated channels (Kir4.1 in lane 3 and Kir4.2 in lane 4) are present, but not in the lanes where no immunoprecipitating antibody was used (lane 1) or where no channel was expressed (lane 2, vector). In Fig. 3, top, a band corresponding to the CaR is present in the third and fourth lanes where the CaR was coimmunoprecipitated with the channels by the anti-Myc antibody.

The next series of experiments demonstrate the selectivity of the interaction between the CaR and Kir4.1 and Kir4.2 based on the failure of the CaR to coimmunoprecipitate with ROMK or Kir5.1. ROMK is predominantly expressed in the kidney where it plays a major role in maintenance of K⁺ homeostasis. Although ROMK activity can be regulated by the CaR (42), it is located in the apical plasma membrane of the thick ascending limb of Henle (TAL) (41, 42) while the CaR is in the basolateral membrane (33). We transiently transfected either Myc-Kir4.2 or ROMK into HEK 293 cells that stably express the CaR, and coimmunoprecipitation was performed using our anti-CaR antibody. Figure 4A demonstrates that both Myc-Kir4.2 and ROMK are expressed at detectable levels in the cell lysates. Figure 4B shows that only Kir4.2 was coimmunoprecipitated with the CaR, although ROMK was strongly expressed in these cells (Fig. 4A). Kir5.1 is expressed in the kidney (23) and may contribute to the basolateral K⁺ conductance by forming heterotetrameric channels with Kir4.1 or Kir4.2 (23, 39, 44). To test for interactions of the CaR with Kir5.1 and interactions of Kir4.2 with Kir5.1, we transiently transfected combinations of different cDNAs (CaR and HA-Kir5.1, CaR and Myc-Kir5.1, CaR and HA-Kir4.2, CaR and Myc-Kir4.2, Myc-Kir4.2 and HA-Kir5.1 or HA-Kir4.2 and Myc-Kir5.1) into HEK 293 cells and then performed coimmunoprecipitation using either our anti-CaR antibody or the polyclonal anti-Myc antibody. In the first two lanes in Fig. 5 where the CaR and Myc-Kir5.1 or the CaR and Myc-Kir4.2 were expressed, no immunoprecipitating antibody was used and no bands are seen when antibodies to the CaR, Myc, or HA were used for Western blotting. In the next four lanes, the anti-CaR antibody was used for immunoprecipitation, and the CaR is present in all four of these lanes (Fig. 5, top). Kir4.2 (HA-Kir4.2 lane 5, and Myc-Kir4.2 lane 6) coimmunoprecipitated with the CaR but Kir5.1 did not (neither HA-Kir5.1 lane 3 nor Myc-Kir5.1 lane 4) (Fig. 5). In Fig. 5, middle and bottom, an IgG band is also present near the top of the panels. In lanes 7–10, the last four lanes, the polyclonal anti-Myc antibody was used for immunoprecipitation. In lane 7, Myc-Kir5.1 is immunoprecipitated (middle), but the CaR is not present (top) because they do not interact. In lane 8, the CaR is coimmunoprecipitated with Myc-Kir4.2 as seen previously. In lane 9, Myc-Kir4.2 and HA-Kir5.1 are both present (middle and bottom, respectively), and conversely in lane 10, Myc-Kir5.1 and HA-Kir4.2 are both present (middle and bottom, respectively), indicating that these two proteins interact. These results demonstrate that the CaR selectively interacts with Kir4.1 and Kir4.2, but not ROMK or Kir5.1, and that Kir4.2 and Kir5.1 interact with each other.

Coexpression of the CaR inactivates Kir4.1 and Kir4.2 channels. Members of the Kir4 subfamily, Kir4.1 and Kir4.2, are expressed in the kidney and may contribute to K⁺ transport in the distal nephron (19, 23). To determine whether the interaction of the CaR with Kir4.1 and Kir4.2 affects channel function, we coexpressed the channels and the CaRWT or the nonfunctional point mutant CaRWT in Xenopus laevis oocytes. Experiments were done 3–7 days after cRNA injections. We tested CaRWT and CaRWT function and expres-

Fig. 3. Reciprocal immunoprecipitation of the CaR with Kir4.1 and Kir4.2 channels. CaR-expressing HEK 293 cells were transiently transfected with vector, Myc-Kir4.1, or Myc-Kir4.2, and immunoprecipitation was performed using antibodies against the CaR or Myc (A-14). The samples were processed for immunoblotting using an anti-CaR, a polyclonal anti-Myc (A-14), or a monoclonal anti-Myc (9E10) antibody to identify the proteins.
sion using Gd$^{3+}$ to activate the endogenous oocyte Cl$^{-}$ channels (transient outward current) (3). Oocytes expressing the CaRW$^{WT}$ alone or with the channels had typical CaR-induced transient outward currents, but the oocytes expressing the CaRR$^{WT}$ mutant did not (representative tracings Fig. 6A, top).

Sweeps of basal currents (98 K$^{+}$ ringer) are shown for water, each of the channels (Kir4.1 and Kir4.2), and each of the receptors (CaRW$^{WT}$ and CaRR$^{WT}$) as well as combinations of receptors and channels (CaRW$^{WT}$ + Kir4.1, CaRW$^{WT}$ + Kir4.2, CaRR$^{WT}$ + Kir4.1 and CaRR$^{WT}$ + Kir4.2). The currents in water-injected oocytes were indistinguishable from the vector-injected oocytes. Currents measured in oocytes expressing K$^{+}$ channels show modest rectification. Coexpression of the CaRW$^{WT}$ with either Kir4.1 or Kir4.2 significantly reduced currents, while coexpression with CaRR$^{WT}$ had no effect on these currents (see figure matrix in Fig. 6A). The magnitude of inactivation was similar in the absence and presence of CaR activation by Gd$^{3+}$ indicating that the effect of the CaR on the channels is tonic in oocytes under these conditions. Figure 6B shows the current-voltage relationships (I-V curves) for the channels alone and coexpressed with CaRW$^{WT}$ or CaRR$^{WT}$. Basal currents for Kir4.1 and Kir4.2 at -120 mV were 8.8 ± 0.8 μA (n = 9) and 9.4 ± 2.3 μA (n = 12), respectively. At -120 mV, coexpression of Kir4.1, and Kir4.2 with CaRW$^{WT}$ leads to current inhibition of 36 and 84%, respectively. Oocyte membranes for these same batches of oocytes were isolated, electrophoresed, blotted, and probed for Kir4.1, Kir4.2 and CaR protein expression. The expression levels of neither Kir4.1 nor Kir4.2 varied with CaR-coexpression (Fig. 6C).

To further define the effect of CaRW$^{WT}$-Kir4 interaction on the activity of channel, we performed coimmunoprecipitation using an anti-Myc antibody and HEK 293 cell lysates that stably coexpressed CaRW$^{WT}$ and Myc-Kir4.2 or CaRR$^{WT}$ and Myc-Kir4.2. As shown in Fig. 7A, expression and membrane distribution of Myc-Kir4.2 were not significantly different in the cells that coexpressed with the CaRW$^{WT}$ or CaRR$^{WT}$. In Fig. 7B, right, the first two lanes are from samples without immunoprecipitating antibody (negative controls). The anti-Myc antibody isolated similar amounts of Myc-Kir4.2 in the cell lysates that coexpressed CaRW$^{WT}$ and Myc-Kir4.2 (lanes 3 and 4) or CaRR$^{WT}$ and Myc-Kir4.2 (lanes 5 and 6), while a band representing the CaR was detectable only in the lanes with the cells that expressed CaRW$^{WT}$ with Kir4.2. These results demonstrate that the nonfunctional mutant CaR, CaRW$^{WT}$, does not interact with Kir4.2 with sufficient affinity to permit coimmunoprecipitation, and with Fig. 6 demonstrate that interaction of the receptor and channel is required for inactivation of Kir4.1/4.2 by the CaR. The presence of high (5.0 mM) Ca$^{2+}$ or low (0.5 mM) Ca$^{2+}$ did not alter the efficiency of immunoprecipitation of the CaRW$^{WT}$ of CaRR$^{WT}$ with the channels (not shown).

Cellular distribution and interaction of the CaR and Kir4 channel in rat kidney. In HEK-293 cells that stably express both the CaR and Myc-Kir4.2, the two proteins were colocalized using our monoclonal anti-CaR antibody (16) and the polyclonal anti-Myc antibody (A-14) as primary antibodies (Fig. 8, A–F). Because both proteins are overexpressed, we observed some cytoplasmic staining. However, the staining is most intense in the plasma membrane and the staining for the CaR and Kir4.2 overlap in this region. Figure 8, G and H, shows staining for the CaR and Kir4.1 in rat kidney sections.
Both proteins are localized to the basolateral membrane of the distal convoluted tubule (DCT).

To demonstrate interaction of the endogenous CaR and endogenous channels from kidney and to verify the specificity of the receptor-channel interactions in renal tissue, we prepared crude membrane and cytosol fractions from rat kidney and processed them for immunoblotting and immunoprecipitation using antibodies against the CaR, Kir4.1, Kir4.2, and ROMK. Specific peptide blocking shows significant levels of CaR, Kir4.2, Kir4.1, and ROMK (Kir1.1) in rat kidney membranes, but little channel or receptor protein in the cytosolic fraction (Fig. 9A). To confirm the interaction of the CaR with Kir4.1 and Kir4.2, we performed coimmunoprecipitation using our anti-CaR antibody for immunoprecipitating with duplicate samples of kidney membrane extracts (Fig. 9B). The first lane of each panel where no immunoprecipitating antibody was present was used as a negative control. A strong band at 130 kDa representing the CaR was immunoprecipitated by our anti-CaR antibody. The bands representing Kir4.2 and Kir4.1 were easily detected, whereas a band representing ROMK was not present (Fig. 9B). These results demonstrate that the endogenous CaR interacts with endogenous Kir4.1 and Kir4.2 in the kidney and that these interactions are selective.

**DISCUSSION**

The goal of the present study was to identify proteins that interact with the CaR in addition to heterotrimeric G proteins and that could contribute to its distinctive effects on transport in the distal nephron. Screening a human adult kidney cDNA library with the COOH terminus of the CaR as bait, we identified Kir4.2, an inwardly rectifying K⁺/H⁺ channel that like the CaR, is expressed in the distal nephron. Since Kir4.1, a closely related channel is also expressed in the distal nephron, we chose to study it along with Kir4.2. We demonstrated that the CaR interacts with Kir4.1 and Kir4.2 when expressed in mammalian cells, but that it does not interact with Kir5.1 or ROMK (Kir1.1), more distantly related channels that are also present in the distal nephron. Both channels are expressed along the basolateral membrane of the distal nephron, and endogenous Kir4.1 and Kir4.2 coimmunoprecipitate with the endogenous CaR from rat kidney membrane extracts. Kir4.1 is...
present in the DCT and cortical collecting duct (CCD), but not the TAL, and Kir4.2 is also expressed in the DCT (19, 23). When expressed in X. laevis oocytes, the CaRWT reduces the currents from Kir4.1 and Kir4.2 regardless of its apparent state of activation, but the CaRR796W does not.

Kir channels are divided into seven subfamilies, Kir1–7, and all tissues express some subfamilies (12). Five subfamilies (Kir1.1, Kir2.3, Kir4, Kir5.1, and Kir7.1) are expressed in the kidney, but the function of only Kir1.1 or ROMK is defined to a significant degree (12, 24, 31, 44). ROMK, the apical small-conductance K⁺ channel, is located in the apical membrane of TAL and other segments and participates in K⁺ recycling across the apical membrane of the TAL for Na⁺ and Cl⁻ reabsorption through the apical NKCC2 transporter. Loss-of-function mutations in this channel result in one form of Bartter’s syndrome. The other Kir channel subfamilies are present in the basolateral membrane (19, 23, 25) where the CaR is also expressed (33). Here, we demonstrate that the CaR interacts with Kir4.1 and Kir4.2 and that they are coexpressed on the basolateral membrane of the DCT, and possibly other segments of the distal nephron. Other investigators have studied the K⁺ conductance of the basolateral membrane of the TAL and DCT (44) and showed that the conductance is ~35–40 pS, pH sensitive, and ATP independent (23), characteristics that are most consistent with homomeric Kir4.1, Kir4.2, or heteromeric Kir4-Kir5.1 channels (28). Since Kir7.1 is also expressed in this region, there are undoubtedly many different conductances composed of a range of proteins, and the molecular identities of the basolateral K⁺ conductances in the distal nephron are not known with certainty.

In addition to controlling second messenger levels, G protein-dependent signaling systems regulate Kir channels by mechanisms that involve protein-protein interactions. Stimulated GPCRs activate Goβγ heterotrimers to release Gβγ subunits that can interact directly with Kir3 channels (designated as GIRK) to activate them, but this mechanism has not been shown to apply to other Kir channels (1, 5, 15, 27, 32, 34). The role of Gα subunits in the regulation of GIRK signaling has been controversial for a number of years (12). Recently, the NH₂ terminus of GIRK2 and the COOH terminus of GIRK1 and GIRK2 were found to interact with the Goα,GDP binding sites (18). RGS proteins that attenuate G protein signaling also inhibit GIRK channel activity, but the molecular basis for this effect is not defined (6, 9). Lavine et al. (21) reported that β₂

Fig. 8. Colocalization of the CaR with Kir4.1 or Kir4.2 in HEK 293 cells and rat kidney. HEK 293 cells that coexpress the CaR and Myc-Kir4.2 were processed for immunofluorescence using our monoclonal anti-CaR antibody (D) and a polyclonal anti-Myc antibody (A-14) (E) as primary antibodies, and Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG as secondary antibodies (overlay, F). A, B, and C: no primary antibodies. Rat kidney sections were immunostained with a polyclonal anti-CaR antibody (G and I) or a polyclonal anti-Kir4.1 antibody (H and J). DCT, distal convoluted tubule; PT, proximal tubule; G, glomerulus.
adrenergic and dopamine D₂ and D₄ receptors interact directly with heteromeric GIRK channels (Kir3.1/3.4 and Kir3.1/3.2), but the physiological consequences of these interactions are not defined. Our results demonstrate a novel mechanism in that the CaR, a GPCR, interacts directly with Kir4.1 and Kir4.2 and inhibits channel function.

Previous studies have shown that the CaR couples to multiple G proteins (Gq, Gq, Gβγ, and Gα12/13) involved in distinct signaling pathways (14). Some of these signaling pathways play an important role in the regulation of ion homeostasis. CaR-activated Gq inhibits the activity of adenylyl cyclase and reduces cAMP accumulation that abolishes hormone-stimulated Na⁺, Cl⁻, and K⁺ transport via the NKCC2 transporter (12). Two other signaling pathways are also involved in the regulation of ROMK functions by the CaR: 1) CaR-stimulated phospholipase A₂ hydrolyzes cellular phospholipids and produces arachidonic acid metabolites which can inhibit ROMK activity (42) and 2) the activity of ROMK is regulated by membrane PIP₂, the hydrolysis and synthesis of which can be regulated by CaR-activated phospholipase C and PKC-kinase (16, 45). Many other GPCRs such as the ANG II receptor, the endothelin receptor, and the adrenergic receptor stimulate similar signaling pathways but do not lead to Na⁺, Cl⁻, and K⁺ wasting as does the CaR. Our finding provides a novel mechanism to explain how the CaR may regulate salt and water balance in the distal nephron.

We do not fully understand how activating mutations of the CaR lead to the Bartter-like syndrome because our understanding of the mechanism of the effects of the CaR on Kir channel activity is incomplete. The inhibitory effect of the CaR on Kir channel activity requires a functional receptor because the CaR(R796W) mutant has no effect. The inhibitory effect of the CaR appears to require interaction of the receptor with the channels because in addition to its lack of effect on channel activity, the CaR(R796W) mutant does not coimmunoprecipitate with the channels. The R796W mutation is in the third intracellular loop, a region of G protein-coupled receptors that interacts with G proteins and so is responsible for second messenger regulation via G proteins. However, this domain also interacts with other signaling proteins such as calmodulin, RGS2, arrestin, receptor kinases, 14–3-3 proteins, Src, and filamin. We do not know which, if any, of these proteins interacts with the third intracellular loop of the CaR, or if the R796W mutation affects more than G protein signaling. Many G protein-coupled receptors are leaky in that they catalyze guanine nucleotide exchange in the absence of agonist. If this were the case for the CaR, persistent PLC activity in the vicinity of the CaR-channel complex could result in reduced levels of PIP₃ and channel inactivation. If this basal rate of guanine nucleotide exchange were of sufficient magnitude, further activation of the receptor with the addition of agonist could have no additional effect on channel activity. Alternatively, the interaction of the CaRW and the channels could reduce the affinity of the channels for PIP₃, having a similar effect. In both of these scenarios, the CaR(R796W) would not be expected to affect channel activity. If in fact the CaR regulates the channels in a tonic, ligand-independent manner, the CaR could have effects of different magnitude if it were expressed at varying levels or if its stoichiometry with respect to the channels varied for any reason. Our functional studies of the CaR-Kir4.1/4.2 channel activity were performed in X. laevis oocytes. Although we expect that in other experimental systems and the intact distal nephron, the CaR would inactivate these channels, other proteins may be present in the CaR-Kir4.1/4.2 complex that would result in a ligand-dependent pattern of regulation of the channels by the CaR. These questions will be addressed in future studies.

Our studies demonstrate that the CaR selectively interacts with Kir4.1 and Kir4.2 in heterologous systems and renal tissue, that they colocalize to the basolateral membrane in the distal nephron, and that this interaction results in the inactivation of Kir4.1 and Kir4.2 currents. These basolateral K⁺ channels are probably involved in K⁺ recycling for the Na⁺-K⁺-ATPase. Inhibition of the basolateral K⁺ conductance in the cortical collecting duct with Ba²⁺ inhibits vasopressin-stimulated Na⁺ transport, so inhibition of basolateral K⁺ channels by a different mechanism should have the same effect (35). The effects of inhibition of basolateral K⁺ channels in the TAL may be more complicated due to the importance of paracellular transport in this segment of the nephron but should reduce Na⁺ transport in the DCT and CCD where both Kir4.1 and Kir4.2 are clearly expressed. By this mechanism in addition to conventional second messenger generation, activation of the CaR which is the combination of CaR-Kir4 interaction and CaR-signaling could significantly inhibit salt and water transport in the distal nephron, consistent with the effects of hypercalcemia and the phenotype of patients with potent activating mutation of the CaR.

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