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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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 ~125 aa from the COOH terminus of the inwardly rectifying K\(^{+}\) channel, Kir4.2. The CaR and Kir4.2 as well as Kir4.1 (another member of Kir4 subfamily) were reciprocally copurified from HEK-293 cells in which they were expressed, but the receptor did not coimmunoprecipitate with Kir5.1 or Kir1.1. Both Kir4.1 and Kir4.2 were immunopurified from rat kidney extracts with the CaR. In Xenopus laevis oocytes, expression of the CaR with either Kir4.1 or Kir4.2 channels resulted in inactivation of whole cell current as measured by two-electrode voltage clamp, but the functional CaR mutant CaR\(^{R706W}\), and that does not coimmunoprecipitate with the channels, had no effect. Kir4.1 and the CaR were colocalized in the basolateral membrane of the distal nephron. The CaR interacts directly with Kir4.1 and Kir4.2 and can decrease their currents, which in turn could reduce recycling of K\(^{+}\) for the basolateral Na\(^{+}\)-K\(^{+}\)-ATPase and thereby contribute to inhibition of Na\(^{+}\) reabsorption.

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 (V\(_{1}\)), the CaR couples to multiple G proteins (Go\(_{i}\), Go\(_{q}\), G\(_{\beta}\gamma\), 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\(_{i}\) with reduced cAMP accumulation inhibits the activity of NCC2 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

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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-Kir4.3 antibody used for immunohistochemistry was obtained from Yoshisahi 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 (CaR\(_{861-1078}\)) 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 leucine, tryptophan, and histidine (37). The plates were replicated on media lacking histidine, tryptophan, leucine, and adenine. The colonies that grew under these conditions were tested for β-galactosidase activity. Yeast expressing the library plasmid pACT2 without an insert and the pACT2 vector with β-galactosidase were used as controls. The positive colonies were analyzed further by purifying the plasmids and sequencing the inserts from either end. The cDNA sequences were analyzed by searching the GenBank database for homology to known DNA sequences. To identify the region(s) of the CaR COOH terminus that interact with Kir4.2, we made the constructs that coded for CaR\(_{860-908}\), CaR\(_{907-997}\), and CaR\(_{987-1078}\) by RT-PCR and subcloned them into pAS2.1. These constructs were mated with the Kir4.2\(_{243-375}\) clone isolated from the cDNA library in pACT2 and plated on medium lacking histidine, leucine, tryptophan, and adenine.

**Construction 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 NH\(_2\) 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 *Eco*RI and NotI and then ligated into *Eco*RI 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 *Eco*RI, blunt-ended, and then digested with KpnI. The Kir5.1pBF construct was digested with *Apa*I, blunt-ended, and then digested with KpnI. The fragments were ligated into pCruz-Myc or -HA constructs were digested with NotI, blunt-ended, and then digested with KpnI. The plasmids were co-transfected with the CaR plasmid pCruz-HA into 293T cells. 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 Kir4.3pCruz, the HA-tagged Kir5.1pCruz, the Myc-tagged Kir5.2pCruz, and the ROMKpcDNA3.1) using the Fu Gene 6 reagent and incubated at 37°C for 24–48 h. All cells were cultured in medium containing 1.8 mM Ca\(_{2+}\). 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 3 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 MgCl\(_2\), 1 mM EDTA, and protease inhibitors, and centrifuged at 2,000 rpm for 10 min. The supernatant was centrifuged at 1,500 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 immunoprecipitated 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 fluorescent microscopy (Zeiss, Model LSM-5 Pascal) and images were collected using the Axiovert 200 program (Zeiss).

For immunoperoxidase staining, kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. The paraffin-
embedded tissue was cut at 2 μm on a rotary microtome (Micron). 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 EGTA 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 × 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 DMRE 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 plasmids were used for in vitro cRNA synthesis. Forty six nanoliters of water or cRNA mixtures were injected into stage VI oocytes (CaR plasmids were used for in vitro cRNA synthesis. Forty six nanoliters were added to oocytes). Oocytes were studied in vivo or in vitro 3–7 days after cRNA injection and were preincubated for 24 h at 10 ng/oocyte, Kir4.1 at 5 ng, Kir4.2 at 5 ng). Oocytes were studied under voltage clamp at −100 to −10 mV. From −100 mV oocytes were stepped by 20 mV from −120 to +80 mV using a 75-ms voltage pulses. I-V curves were generated by averaging the final 10 ms of each resulting current responses as previously described (8, 36). We verified the activation of the CaR by monitoring current responses (endogenous oocyte Cl channel) after the addition of 1 mM GdCl₃ (3). Only the wild-type CaR (CaR⁶⁶⁰–¹⁰⁷⁸) showed an outward current with Gd³⁺ addition. Subsequently, we used 20–30 sister oocytes to isolate membranes to document the CaR and Kir4.x protein expression.

**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 (CaR⁶⁶⁰–¹⁰⁷⁸) 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 (CaR⁶⁶⁰–⁹⁰⁸, CaR⁹⁰⁷–⁹⁹⁷, 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 only interacts with the CaR⁹⁰⁷–⁹⁹⁷ 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 immunoprecipitation 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 CaR796W in Xenopus laevis oocytes. Experiments were done 3–7 days after cRNA injections. We tested CaRWT and CaR796W function and expres-

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**Fig. 2.** Commonmunoprecipitation of the CaR and Kir4.2. A: HEK 293 cells that stably express the CaR were transiently transfected with vector (CaR + V) or Myc-Kir4.2 (CaR + Kir4.2). HEK-293 cells that express neither protein were used as negative controls. Protein expression was documented in the cell extracts by immunoblotting using antibodies against the CaR (left) and Myc (right). B: immunoprecipitation was performed using the anti-CaR antibody, and the samples were processed for immunoblotting using the anti-CaR antibody or an anti-Myc antibody (A-14). IP-AB, immunoprecipitating antibody; WB-AB, Western blotting antibody; No, IP-AB was not used.

**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.

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**Fig. 4.** A: Western blotting analysis to determine the expression of CaR, Myc, and Kir4.2. B: Immunoprecipitation of the CaR and Kir4.2. The CaR and Myc-Kir4.2 or ROMK 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 immuno-precipitated (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 CaR796W in Xenopus laevis oocytes. Experiments were done 3–7 days after cRNA injections. We tested CaRWT and CaR796W function and expres-
sion using Gd\(^{3+}\) to activate the endogenous oocyte Cl\(^{-}\) channels (transient outward current) (3). Oocytes expressing the CaR\(^{WT}\) alone or with the channels had typical CaR-induced transient outward currents, but the oocytes expressing the CaR\(^{R796W}\) 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 (CaR\(^{WT}\) and CaR\(^{R796W}\)) as well as combinations of receptors and channels (CaR\(^{WT}\) + Kir4.1, CaR\(^{WT}\) + Kir4.2, CaR\(^{R796W}\) + Kir4.1 and CaR\(^{R796W}\) + 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 CaR\(^{WT}\) with either Kir4.1 or Kir4.2 significantly reduced currents, while coexpression with CaR\(^{R796W}\) 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 CaR\(^{WT}\) or CaR\(^{R796W}\). Basal currents for Kir4.1 and Kir4.2 at -120 mV were 8.8 ± 0.8 \(\mu\)A (n = 9) and 9.4 ± 2.3 \(\mu\)A (n = 12), respectively. At -120 mV, coexpression of Kir4.1, and Kir4.2 with CaR\(^{WT}\) 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). Thus a functional CaR protein is necessary for Kir4.1 and Kir4.2 channel inactivation. These results demonstrate that interaction of the CaR\(^{WT}\) with Kir4.1 or Kir4.2 results in tonic partial inactivation of both channels but that a signaling-defective mutant CaR has no effect on channel activity.

To further define the effect of CaR\(^{WT}\)-Kir4 interaction on the activity of channel, we performed coimmunoprecipitation using an anti-Myc antibody and HEK 293 cell lysates that stably coexpressed CaR\(^{WT}\) and Myc-Kir4.2 or CaR\(^{R796W}\) 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 CaR\(^{WT}\) or CaR\(^{R796W}\). 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 CaR\(^{R796W}\) with Myc-Kir4.2 (lanes 3 and 4) or CaR\(^{WT}\) 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 CaR\(^{WT}\) with Kir4.2. These results demonstrate that the nonfunctional mutant CaR, CaR\(^{R796W}\), 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 CaR\(^{WT}\) of CaR\(^{R796W}\) 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.

Fig. 5. Reciprocal immunoprecipitation of the CaR and Kir4.2 but not Kir5.1. HEK 293 cells were transiently transfected with the different plasmids (CaR and Myc-Kir5.1, CaR and Myc-Kir4.2, CaR and HA-Kir5.1, CaR and HA-Kir4.2, Myc-Kir5.1 or Myc-Kir5.1 and HA-Kir4.2). The immunoprecipitation was performed using antibodies against the CaR or Myc, and then the samples were processed for immunoblotting using antibodies against the CaR, Myc, or HA to identify the proteins.

Fig. 4. Coimmunoprecipitation of the CaR and Kir4.2 but not ROMK. CaR-expressing HEK 293 cells were transiently transfected with Myc-Kir4.2 or ROMK. The cell lysates were processed for immunoblotting using antibodies against ROMK and Myc to identify the expressed proteins (A). The immunoprecipitations were performed using our anti-CaR antibody and were processed for immunoblotting using antibodies against the CaR, Myc, or ROMK (B).
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 immunoprecipitation 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 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 heterotrimer 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
adrenergic and dopamine D$_2$ and D$_4$ 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 (G$_{ot}$, G$_{otq}$, G$_{ab}$, and G$_{ot2/3}$) involved in distinct signaling pathways (14). Some of these signaling pathways play an important role in the regulation of ion homeostasis. CaR-activated G$_{ot}$ 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 A$_2$ hydrolyzes cellular phospholipids and produces arachidonic acid metabolites which can inhibit ROMK activity (42) and 2) the activity of ROMK is regulated by membrane PIP$_2$, the hydrolysis and synthesis of which can be regulated by CaR-activated phospholipase C and PI-4 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 transport 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 PIP$_2$ 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 CaR$^{WT}$ and the channels could reduce the affinity of the channels for PIP$_2$, 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 Ba$^{2+}$ 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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