The agonist-binding Domain of the Calcium-sensing Receptor Is Located at the Amino-terminal Domain*

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The calcium-sensing receptor (CaR) is a G-protein-coupled receptor that displays 19–25% sequence identity to the γ-aminobutyric acid type B (GABA_B) and metabotropic glutamate (mGlu) receptors. All three groups of receptors have a large amino-terminal domain (ATD), which for the mGlu receptors has been shown to bind the endogenous agonist. To investigate whether the agonist-binding domain of the CaR also is located in the ATD, we constructed a chimeric receptor named CaR1a consisting of the ATD of CaR and the seven transmembrane region and C terminus of mGlu1a. The CaR1a receptor stimulated inositol phosphate production when exposed to the cationic agonists Ca2+, Mg2+, and Ba2+ (for the latter two cations, respectively) very similar to that of the wild-type CaR (EC50 values of 3.3, 2.6, and 3.9 mM, respectively). For the mGlu1a receptor, it has been shown that Ser-165 and Thr-188, which are located in the ATD, are involved in the agonist binding. An alignment of CaR with the mGlu receptors showed that these two amino acid residues have been conserved in CaR as Ser-147 and Ser-170, respectively. Each of these residues was mutated to alanines and tested pharmacologically using the endogenous agonist Ca2+. CaR-S147A showed an impaired function as compared with wild-type CaR both with respect to potency of Ca2+ (4-fold increase in EC50) and maximal response (79% of wild-type response). CaR-S170A showed no significant response to Ca2+ even at 50 mM concentration. In contrast, each of the two adjacent mutations, S169A and S171A, resulted in pharmacological profiles almost identical to that of the wild-type receptor. These data demonstrate that Ser-170 and to some extent Ser-147 are involved in the Ca2+ activation of the CaR, and taken together, our results reveal a close resemblance of the activation mechanism between the CaR and the mGlu receptors.

The cloning of the gene coding for the calcium-sensing receptor (CaR1 (1, 2)) has revealed that it together with the γ-aminobutyric acid type B (GABA_B) (3–6) and metabotropic glutamate (mGlu) receptors (7, 8) form a separate family (family 3) within the superfamily of seven transmembrane G-protein-coupled receptors (GPCR). A distinctive property of this receptor family is the unusually large amino-terminal domain (ATD) on the extracellular side of the membrane. However, the amino acid sequence identities between the three receptor classes within family 3 is relatively low as exemplified by the CaR, which shows approximately 25% sequence identity to the mGlu1a receptor (2).

Previously we have shown that the ATD of mGlu1a is related to bacterial periplasmic binding proteins (PBPs), and based on the crystal structure of the Leu/Ile/Val binding protein (LIVBP) we developed a molecular model of the glutamate-binding domain of the mGlu1a receptor (9). As predicted by this model, we have shown that glutamate binding was significantly reduced by mutations of Ser-165 and/or Thr-188 in mGlu1a, which align with Ser-79 and Thr-102, respectively, which bind the amino acid ligand in LIVBP (9, 10). The glutamate binding to the ATD has been confirmed by a series of other experiments. Thus, it has been shown that agonist selectivity can be interchanged by generating chimeric receptors within the mGlu receptor family in which the ATDs are interchanged (11, 12). Furthermore, it has been shown that antibodies raised against the ATD antagonize agonist responses (13), and finally it has recently been shown that the purified soluble ATD of a truncated mGlu1a receptor displays pharmacological characteristics similar to the wild-type receptor (14).

Recently, mGlu receptors have been shown to be modulated by Ca2+ and other polyvalent cations (15, 16). By use of chimeric receptors, it was shown that this modular site is situated in the ATD, and point mutations located the site of action to Ser-166 in mGlu1a (15). Although it is at present unclear whether Ca2+ acts as a direct agonist (15) or a co-agonist (16), it is interesting to note the pharmacological similarity to the CaR. However, in contrast to the mGlu receptors very little is known about the site of action of agonists on the CaR, and the aim of the present study was to investigate whether the results of structure-function studies on the mGlu receptors could be applied to the CaR.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were obtained from Sigma. Culture media, serum, antibiotics, and buffers for cell culture were obtained from Life Technologies, Inc. (Paisley, United Kingdom). The plasmids CaR-pRK5 and pmGR1 were generous gifts from Professor Solomon H. Snyder.

GPCR, G-protein-coupled receptor; HBSS, Hanks’ balanced saline solution; HEK, human embryonic kidney; IP, inositol phosphate; LEUBP, leucine-binding protein; LIVBP, Leu/Ile/Val-binding protein from Escherichia coli; LIVPA, Leu/Ile/Val-binding protein from Pseudomonas aeruginosa; mGlu, metabotropic glutamate; GABA_B, γ-aminobutyric acid type B; PBP, periplasmic binding protein.
(The Johns Hopkins University School of Medicine, Baltimore, MD) and Professor Shigetada Nakanishi (Kyoto University, Kyoto, Japan). The pSI vector was obtained from Promega (Madison, WI). The tsA cells were a generous gift from Dr. Penelope S. V. Jones (University of Vermont, Burlington, VT).

Construction of Chimeric and Point-mutated Receptors—The rat CaR was transferred from the pRK5 vector to the pSI vector using the unique flanking restriction sites XhoI and XbaI. Similarly, the rat mGlu4 receptor was transferred to the pSI vector by a partial digest using the flanking restriction sites EcoRI and XbaI. A silent DraIII site was engineered into mGlu4-pSI at amino acid 577 and into CaR-pSI at amino acid 597. These DraIII sites are placed 16 and 17 amino acids upstream of the predicted first transmembrane region of the CaR and mGlu4 receptors, respectively (1, 17). The chimeric receptor named Ca/1a was generated by cutting out the DraIII-XbaI fragment of CaR-pSI and replacing it with the DraIII-XbaI fragment of mGlu4-pSI. By this strategy all cysteines of the cysteine-rich domain in the ATD were left intact. The point mutations S147A, S169A, S170A, and S171A were introduced in CaR-pRK5, and the XhoI-EcoRI fragments containing the mutations were transferred to CaR-pSI. Mutations were made using the Quickchange mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA), and all amplified receptor DNAs were sequenced on an ABI 310 using the Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Warrington, UK).

Cell Culture and Second Messenger Assay—tsA cells (a transformed HEK 293 cell line (18)) were maintained at 37°C in a humidified 5% CO2 incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and 10% fetal calf serum. One million cells were split into a 10-cm tissue culture plate and transfected with 5 μg of plasmid the following day using SuperFect as a DNA carrier according to the protocol by the manufacturer (Qiagen, Hilden, Germany). The day after transfection, cells were split into a poly-D-lysine-coated 24-well tissue culture plate in 0.5 ml DMEM (containing antibiotics, 10% fetal calf serum, and 2 mM L-glutamine) and incubated at 37°C for 20 min. Buffer was removed, and cells were incubated for 20 min in the same buffer containing 0.5 mM CaCl2, 0.5 mM MgCl2, and 10 mM LiCl. Buffer was removed, and cells were incubated for 20 min in the same buffer containing the indicated ions. The reactions were stopped by exchanging the buffer with 0.5 ml of 20 mM ice-cold formic acid, and separation of total [3H]inositol phosphates was carried out by ion-exchange chromatography as described previously (19).

Data Analysis—All experiments were performed in triplicate, and R is response. Curves were generated by non-weighted least-squares fits using the program KaleidaGraph 3.08 (Synergy Software, Reading, PA).

RESULTS
Pharmacology of Wild-type CaR and Chimeric Ca/1a Receptors Expressed in tsA Cells—As shown in Fig. 1, the cationic agonists Ca2+, Mg2+, and Ba2+ were stimulating IP accumulation in tsA cells transiently transfected with CaR-pSI. In agreement with previous studies, Mg2+ was slightly less potent than the equipotent Ca2+ and Ba2+ (Table I (1, 20)). Mg2+ and Ba2+ have previously been shown to be partial and full agonists, respectively, as compared with Ca2+ (20). In agreement with this study, we find Mg2+ to be a partial agonist with an intrinsic activity of 69 ± 5%, but in the present studies, Ba2+ was shown to also act as a partial agonist with an intrinsic activity of 49 ± 6%. Ca2+ and Ba2+ both show Hill numbers of approximately 4 whereas Mg2+ shows a significantly lower value of 2.3 ± 0.3 (Table I) as has previously been shown by others (20). Cells transfected with the pSI vector alone did not show any response to the applied maximal concentration of agonists (data not shown).

To determine the binding domain of cationic agonists on CaR, we exchanged the ATD of mGlu4-pSI with the ATD of CaR thus constructing the chimeric receptor named Ca/1a-pSI. When tsA cells were transiently transfected with Ca/1a, the cells stimulated IP production when exposed to increasing concentrations of the agonists Ca2+, Mg2+, and Ba2+ in a manner very similar to cells transfected with wild-type CaR (Fig. 1). Comparison of the pharmacological parameters of the three agonists tested using the chimeric Ca/1a and wild-type CaR reveals a very similar pharmacological profile, in terms of potency and intrinsic activity (Table I). Interestingly the Hill

![FIG. 1. Agonist concentration-response curves for wild-type and chimeric CaRs. Concentration-response curves of agonist-induced IP stimulation (expressed as disintegrations per minute (DPM) per 24-cluster well) in tsA cells transfected with wild-type CaR (A) or the chimeric Ca/1a receptor (B). Cells were prelabeled overnight with 2 μCi/ml [3H]inositol phosphates was carried out by ion-exchange chromatography as described previously (19).](image)

**Table I** Pharmacological parameters for cationic agonists at wild-type CaR and the chimeric Ca/1a receptor expressed in tsA cells

| Agonist | CaR | Ca/1a |
|--------|-----|------|
|        | EC50 | Response | Hill |
| Ca2+   | 3.2 ± 0.1 | 100 | 3.6 ± 0.3 | 3.3 ± 0.2 | 100 | 2.1 ± 0.2 |
| Mg2+   | 4.7 ± 0.1 | 69 ± 5 | 2.3 ± 0.3 | 2.6 ± 0.4 | 72 ± 4 | 2.2 ± 0.5 |
| Ba2+   | 4.1 ± 0.1 | 48 ± 3 | 4.3 ± 0.3 | 3.9 ± 0.2 | 53 ± 2 | 2.7 ± 0.4 |
numbers for Ca\(^{2+}\) and Ba\(^{2+}\) were reduced to 2.1 ± 0.2 and 2.7 ± 0.4, respectively, whereas Mg\(^{2+}\) showed a Hill number similar to the CaR value.

**Pharmacology of Point-mutated CaRs**—For the mGlu1 receptor, it has previously been shown that Ser-165 and Thr-188 play a key role in the binding of L-glutamic acid (9) and that Ser-166 is involved in binding of the modulatory Ca\(^{2+}\) ions (15).

When the ATD of the rat CaR is aligned with the PBPs and the eight cloned rat mGlu receptors, it can be seen that Ser-147 and Ser-170 in rat CaR correspond to Ser-165 and Thr-188, respectively, of mGlu1 (see Fig. 2). Based on the involvement of a serine in the binding of the modulatory Ca\(^{2+}\) ion in mGlu1, we performed each of the point mutations S147A and S170A in the rat CaR to test whether these amino acid residues are involved in the activation of CaR by Ca\(^{2+}\). In addition, we made each of the adjacent CaR point mutations, S169A and S171A. The concentration-response curves for the point-mutated CaRs are shown in Fig. 3, and the derived pharmacological parameters are shown in Table II. The S147A mutation attenuated the receptor, lowering the potency of the endogenous agonist Ca\(^{2+}\).
approximately 4-fold and reducing the maximal response to 79 ± 13% of the wild-type response. A much more dramatic effect was seen in the S170A-mutated CaR, which was unable to respond to Ca\(^{2+}\) even at a concentration of 50 mM. In striking contrast, the adjacent mutation S169A provided a CaR with a pharmacological profile identical with that of the wild-type CaR. The other adjacent mutation, S171A, also resulted in a CaR with a pharmacological profile close to the wild-type CaR, although with a 2-fold reduction of Ca\(^{2+}\) potency (Table II).

**DISCUSSION**

The ATD of mGlu receptors has previously been subjected to structure-function studies, which have disclosed that for mGlu1, the endogenous agonist glutamate binds to Ser-165 and Thr-188 in the ATD (9). Furthermore, it has recently been shown that Ca\(^{2+}\) modulates the mGlu1 receptor (15, 16) and that the modulatory site is also located in the ATD at Ser-166 (15). On the other hand, very little is known about the site of action of the cationic agonists activating the CaR. In this paper we have thus used chimeric and point-mutated receptors to study whether the results of structure-function studies on the mGlu receptors could be transferred to the CaR.

As part of these studies, we generated a chimeric receptor named Ca/1a consisting of the ATD of CaR and the transmembrane region and cytoplasmic tail of mGlu1a. Interestingly, this chimeric receptor expressed a pharmacological profile almost identical to that of the wild-type CaR. This clearly demonstrates that the ATD is the site of action of cationic agonists at the CaR in agreement with results from the mGlu receptors (11, 12). In light of the relatively low overall sequence identity of 19–25% between the CaR and the mGlu receptors (1, 2), it is surprising that the chimeric receptor is functioning so well. At this point, it is not known how the signal from the first messenger is transduced from the ATD to the intracellular loops that are responsible for G-protein activation (21). In any case, our results show that this mechanism of action, of unknown nature, has been conserved even between distantly related members of family 3 of GPCRs characterized by large agonist binding ATDs. The only significant difference between CaR and Ca/1a was the decreased Hill number for Ca\(^{2+}\) and Ba\(^{2+}\) at the chimeric receptor compared with the wild-type receptor. Different explanations have been suggested for the unusually high Hill number for Ca\(^{2+}\) and other cations at CaR (20, 22): 1) multiple Ca\(^{2+}\) binding sites; 2) interactions between CaRs such as dimerization; or 3) ligand-dependent cooperativity between CaR and G-proteins. Recently it was shown that a region of the intracellular C terminus of CaR close to the transmembrane domain (amino acids 868–886) is important for the observed cooperativity of, for example, Ca\(^{2+}\) since removal of this region significantly reduces the Hill number (22). The chimeric receptor we constructed contains the C-terminal of mGlu1a, and since there is no sequence homology between CaR and mGlu1a in the C-terminal region close to the transmembrane domain (22) this could offer an explanation for the decreased Hill numbers for Ca\(^{2+}\) and Ba\(^{2+}\) that we observed for Ca/1a compared with CaR.

To study the site of action of the endogenous agonist Ca\(^{2+}\) in the ATD of the CaR, we performed an alignment of the CaR with the PBP's and the mGlu receptors. Interestingly, Ser-165 and Thr-188, which have been shown to bind glutamate to mGlu1 (9), have been conserved as Ser-147 and as the conserving mutations on mGlu1, we found that CaR-S147A is functionally impaired, whereas CaR-S170A is totally unable to respond to the endogenous agonist Ca\(^{2+}\). We also mutated each of the adjacent serines at positions 169 and 171 in CaR to alanines. These mutated receptors have pharmacological profiles identical to, or slightly impaired, relative to the wild-type receptor, respectively. The lack of activity of the S170A mutated CaR could be caused by disturbance of the protein folding. On the other hand, since the similar amino acid replacement of the adjacent amino acid (S169A) had no effect on the function of the receptor, we do not find it very likely that loss of a single OH group in this region should be crucial for correct folding of the protein. Thus, our conclusion is that Ser-170 and, to some extent, Ser-147 are important for agonist activation of CaR by the endogenous agonist Ca\(^{2+}\).

A number of proteins for which the three-dimensional structure has been determined by x-ray crystallography bind Ca\(^{2+}\) ions, but only rarely has a serine been shown to act as a Ca\(^{2+}\) ligand in these proteins, presumably due to the lack of additional functional groups in the serine side chain as participants in the stabilization of hydrogen bond networks (23). In this light, it is interesting to note the importance of serine residues in mediating the effects of Ca\(^{2+}\) on both mGlu1 and CaR. In the protein structures so far studied by x-ray crystallography, the Ca\(^{2+}\) coordination numbers are 5–8. Thus, it should be expected that residues other than Ser-147 and Ser-170 are acting as Ca\(^{2+}\) ligands in the CaR. Furthermore, as mentioned previously, multiple Ca\(^{2+}\) ions might bind to the receptor, which would even further increase the number of residues acting as Ca\(^{2+}\) ligands in the receptor. It has recently been shown that serines in the GABA\(_{B1}\) receptor corresponding to Ser-147 and Ser-170 in CaR and to Ser-165 and Thr-188 in mGlu1 are important for binding of ligands to the GABA\(_{B1}\) receptor (24).

**TABLE II**

Pharmacological parameters for the agonist Ca\(^{2+}\) at wild-type and point-mutated CaRs expressed in tsk cells

| Receptor    | EC\(_{50}\) | Response | Hill |
|-------------|------------|----------|------|
| CaR-wt      | 3.2 ± 0.1  | 100      | 3.6 ± 0.3 |
| CaR-S147A   | 12.1 ± 0.1 | 79 ± 13  | 2.7 ± 0.1 |
| CaR-S169A   | 2.7 ± 0.2  | 92 ± 10  | 3.3 ± 0.3 |
| CaR-S170A   | >20        | <10      | n.d.  |
| CaR-S171A   | 7.0 ± 0.1  | 118 ± 14 | 3.8 ± 0.6 |
Thus, in conclusion it appears that all of the mammalian receptors cloned so far that belong to family 3 of GPCRs with a large ATD bind their agonists in a similar fashion in the ATD, and that the signal is subsequently transmitted to the intracellular site of the membrane by an as yet unknown mechanism which, however, appears also to be conserved within this subfamily of GPCRs.

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