

Domains Determining Ligand Specificity for Ca^{2+} Receptors

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ABSTRACT

The Ca^{2+} receptor is a G protein-coupled receptor that enables parathyroid cells and certain other cells in the body to respond to changes in the level of extracellular Ca^{2+} . The Ca^{2+} receptor is a member of a family of G protein-coupled receptors that includes metabotropic glutamate receptors (mGluRs), γ -aminobutyric acid_B receptors, and putative pheromone receptors. As a family, these receptors are characterized by limited sequence homology and an unusually large putative extracellular domain (ECD). The ECD of the mGluRs is believed to determine agonist selectivity, but the functions of the structural domains of the Ca^{2+} receptor are not known. To identify structural determinants for cation recognition and activation of the Ca^{2+} receptor (and to further study the mGluRs), two chimeric receptors were constructed in which the large ECD of the Ca^{2+} receptor and the mGluR1 were interchanged. When expressed in *Xenopus laevis* oocytes, one of these chimeras, named CaR/

mGluR1 [ECD of the Ca^{2+} receptor and transmembrane domain (TMD) of the mGluR1], responded to cation agonists (Gd^{3+} , Ca^{2+} , neomycin) of the Ca^{2+} receptor at concentrations similar to those necessary for activation of the native Ca^{2+} receptor. A reciprocal construct, named mGluR1/CaR (ECD of the mGluR1 and TMD of the Ca^{2+} receptor), was responsive to mGluR agonists but was much less sensitive to two of three cation agonists known to activate the Ca^{2+} receptor. A deletion construct of the Ca^{2+} receptor (ΔntCaR), which lacked virtually the entire ECD, was only activated by one of three agonists tested. These results suggest that the primary determinants for agonist activation of both the Ca^{2+} receptor and the mGluRs are found in the large ECD and that the Ca^{2+} receptor is possibly distinguished from the mGluRs in that it may contain sites in the TMD that permit activation by certain cation agonists.

Systemic Ca^{2+} homeostasis is regulated by several mechanisms. Principal among these mechanisms is the regulation of parathyroid hormone secretion by a G protein-coupled receptor (GPCR) known as the Ca^{2+} receptor, which enables parathyroid cells and certain other cells to respond to changes in extracellular Ca^{2+} concentrations (Brown et al., 1993; Garrett et al., 1995). Elevated levels of plasma Ca^{2+} activate the Ca^{2+} receptor, thereby inhibiting parathyroid hormone secretion and ultimately reducing serum Ca^{2+} levels (Nemeth and Scarpa, 1986; Brown, 1991). The Ca^{2+} receptor is also responsive in vitro to a variety of inorganic and organic polycations other than Ca^{2+} , including gadolinium (Gd^{3+}), magnesium (Mg^{2+}), and neomycin.

The Ca^{2+} receptor is a member of a structurally related family of GPCRs that includes the metabotropic glutamate receptors (mGluRs), the γ -aminobutyric acid_B receptors, and putative pheromone receptors. This family is structurally unique because its members share little or no homology with most other known GPCRs and only limited sequence homology (~20% amino acid identity) with each other (Herrada and Dulac, 1997; Kaupman et al., 1997; Ryba and Tirindelli, 1997). These receptors contain a large putative extracellular domain (ECD) that consists of about 600 amino acids, and

earlier studies of mGluRs showed that this large extracellular domain determined the rank order of potency for certain mGluR agonists (Takahashi et al., 1993).

The structural determinants responsible for agonist binding and subsequent activation of the Ca^{2+} receptor are not known. Ca^{2+} has a relatively low apparent affinity for the Ca^{2+} receptor ($\text{EC}_{50} \approx 1.4$ mM, in vitro), which suggests that the Ca^{2+} receptor lacks the structural characteristics (such as consensus binding sequences) that are found within the numerous intracellular proteins that bind Ca^{2+} with much higher affinity (Persechini et al., 1989). The Ca^{2+} receptor contains highly acidic regions in the ECD and an acidic segment in the second extracellular loop of the seven transmembrane domains (TMD), and it has been proposed that either or both of these regions could be cation recognition sites (Brown et al., 1993; Garrett et al., 1995). Mutations of the Ca^{2+} receptor are responsible for a condition called familial hypocalciuric hypercalcemia, which is an autosomal dominant disorder that causes abnormal elevations in serum Ca^{2+} concentrations. Exogenous expression of these mutant receptors has shown a general reduction or loss of sensitivity to agonists (Brown, 1997). Unfortunately, these mutations are found throughout the receptor and have therefore pro-

ABBREVIATIONS: GPCR, G protein-coupled receptor; mGluR, metabotropic glutamate receptor; ECD, extracellular domain; TMD, transmembrane domain; PCR, polymerase chain reaction; CaR, calcium receptor.

Results

Agonist Responsiveness of Ca²⁺ Receptor and mGluR1a. The native mGluR1a and the Ca²⁺ receptor were responsive to agonists at concentrations similar to those previously reported for each receptor. In each case, agonists were found to be mostly receptor selective. The Ca²⁺ receptor was activated by Ca²⁺, Gd³⁺, or neomycin, but not by glutamate or quisqualate. The mGluR1a was activated by quisqualate and by L-glutamate, but cations were typically ineffective. Previous reports have demonstrated that certain mGluRs (including mGluR1a) are responsive to cation agonists of the Ca²⁺ receptor (Kubo et al., 1998). Responses to certain cation agonists were also noted in the present study. A specific example was the activation of mGluR1a by Gd³⁺. Curiously, these particular responses varied from oocyte to oocyte and from batch to batch. They were most prominent when mGluR1a responses to L-glutamate were unusually large (data not shown). Ca²⁺ and neomycin, however, were unable to elicit responses under these conditions. When mGluR1 agonists were tested, quisqualate was the more potent agonist of the mGluR1 compared with L-glutamate. Taken together, these results are consistent with those described previously for the agonist concentration-response characteristics of the Ca²⁺ receptor and the mGluR1a (Masu et al., 1991; Brown et al., 1993; Takahashi et al., 1993). Figure 1 shows representative current traces of Ca²⁺ receptor and mGluR1a activation in oocytes by their respective agonists.

Agonist Responsiveness of the CaR/mGluR1. A chimeric receptor containing the putative ECD of the Ca²⁺ receptor and the TMD and cytoplasmic tail of the mGluR1a (named CaR/mGluR1) was assessed for agonist sensitivity after expression in *X. laevis* oocytes. CaR/mGluR1 responded to Ca²⁺, Gd³⁺, or neomycin at concentrations that were very similar to those necessary for activation of the native Ca²⁺ receptor. CaR/mGluR1 failed to respond to glutamate or quisqualate, even at concentrations as high as 1 mM (Fig. 2). Further evaluation of agonist responsiveness and a comparison of the agonist concentration-response characteristics of the Ca²⁺ receptor and the CaR/mGluR1 show that the rank order of potency of the three cations did not differ from their potencies on the native Ca²⁺ receptor. Gd³⁺ was the most potent agonist of the CaR/mGluR1, followed by neomycin, then Ca²⁺ (Fig. 3).

Agonist Responsiveness of the mGluR1/CaR. A reciprocal chimeric receptor containing the ECD of the mGluR1 and the TMD and cytoplasmic tail of the Ca²⁺ receptor (named mGluR1/CaR), was expressed in oocytes and assessed for agonist responsiveness. The mGluR1/CaR was responsive to the glutamate receptor agonists quisqualate or glutamate at concentrations similar to those necessary for activation of the native mGluR1 (Fig. 4). In addition, mGluR1/CaR was responsive to certain CaR agonists. Gd³⁺ was by far the most potent of the cation agonists and it consistently activated the receptor at concentrations even lower than necessary for activation of the native Ca²⁺ receptor (Fig. 4). Neomycin and Ca²⁺, however, were much less effective on the mGluR1/CaR than on the native Ca²⁺ receptor and seemed unable to fully activate the chimeric mGluR1/CaR (data not shown).

vided limited insight into the site(s) of action for agonists of the Ca²⁺ receptor. To identify domains necessary for cation activation of the Ca²⁺ receptor, two chimeric receptors were constructed in which the large ECD of the Ca²⁺ receptor and mGluR1 were interchanged. In addition to these two chimeras, a deletion mutant of the Ca²⁺ receptor was made that lacked virtually the entire ECD. These constructs were analyzed after expression in *Xenopus laevis* oocytes. The results of these studies indicate that the Ca²⁺ receptor, like the mGluRs, contains determinants for agonist activation that reside in the ECD. Unlike the mGluRs, the Ca²⁺ receptor seems to differ functionally in that it possesses domains that enable activation by certain cation agonists that also seem to be contained in the TMD.

Materials and Methods

Construction of Chimeric and Deletion Mutant Receptors. The mGluR1a (Masu et al., 1991) used in these studies was isolated from a rat olfactory bulb cDNA library (Stratagene, La Jolla, CA) screened with rat mGluR1 specific 5' and 3' oligonucleotides. The chimeric receptors and the amino-terminal deletion mutant were constructed by polymerase chain reaction (PCR) (Horton et al., 1989). The mGluR1/CaR chimera encodes the extracellular domain of rat mGluR1, corresponding to amino acids 1 to 592, which are spliced to the transmembrane domain and cytoplasmic tail of the human Ca²⁺ receptor at amino acid 613 and thus contain the transmembrane domain and intracellular region of the Ca²⁺ receptor corresponding to amino acids 613 to 1078. The CaR/mGluR1 chimera was made to encode a protein containing the putative ECD of the Ca²⁺ receptor (amino acids 1 to 598) and the TMD and cytoplasmic tail of the rat mGluR1 (amino acids 579 to 1199). The amino-terminally deleted Ca²⁺ receptor is an epitope-tagged expression construct (Δ ntCaR) that encodes a protein consisting of the first 22 amino acids of the native Ca²⁺ receptor protein (the putative signal sequence) linked to a 9-amino-acid epitope tag (YPYDVPDYA) (Green et al., 1982), followed by the human Ca²⁺ receptor protein from amino acid 600 to the carboxyl terminus (amino acid 1078). This construct lacks the majority of the 610 amino acid extracellular domain of the Ca²⁺ receptor, and therefore contains only the TMD and the carboxyl-terminal intracellular domain. All junctions and recombinant DNA sequences were confirmed by double-stranded DNA sequencing.

RNA Transcription and Oocyte Expression. RNA was transcribed as described previously (Garrett et al., 1995) and dissolved in water. Individual oocytes were injected with 50 nl of the cRNA solution (12.5 ng/oocyte). After injection, oocytes were incubated at 16°C in modified Barth's saline containing 0.5 mM CaCl₂, 100 U/ml penicillin, and 100 μ g/ml streptomycin for 2 to 5 days before assay.

Two-Electrode Voltage-Clamp and Concentration-Response Studies. Oocytes were voltage-clamped at a holding potential of -60 mV with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) using standard two-electrode voltage-clamp techniques (Racke et al., 1993). Currents were recorded on a chart recorder. The standard control saline was ND96, which contained 96 mM NaCl, 4 mM KCl, 10 mM HEPES, pH 7.5, 0.3 mM CaCl₂ and 0.8 mM MgCl₂. Test substances were dissolved in ND96 and applied by superfusion at a flow rate of about 5 ml/min. All experiments were done at room temperature. Activation of the endogenous calcium-activated chloride current (I_{Cl}) was quantified by measuring the peak inward current evoked by the agonist, relative to the holding current at -60 mV. Increases in I_{Cl} were measured in response to the application of agonists. A curve was fit to the data from all experiments with the Levenberg-Marquardt algorithm using the Kaleidograph fitting program (Synergy Software, Reading, MA).

Pharmacological Characterization of the Δ ntCaR. The responsiveness of the mGluR1/CaR to Gd^{3+} suggested that functional determinants necessary for agonist interaction with the Ca^{2+} receptor might be contained in the TMD. To address this hypothesis, an amino-terminal deletion mutant of the CaR was constructed that lacked virtually the entire ECD of the Ca^{2+} receptor, including the acidic amino-acid-containing domains of the amino-terminal ECD that might interact with cation agonists. This deletion mutant receptor, Δ ntCaR, did not respond to the application of Ca^{2+} (up to 20 mM), and showed only very small responses to neomycin at concentrations >1 mM. However, the Δ ntCaR was activated by Gd^{3+} at concentrations only about 3-fold higher than those necessary for activation of the native Ca^{2+} receptor or the CaR/mGluR1 ($\text{EC}_{50} = 70 \mu\text{M}$ versus $25 \mu\text{M}$) (Fig. 5).

Gd^{3+} Concentration-Response Analysis of the Chimeric and Mutant Receptors. Gd^{3+} was the most potent agonist tested and it activated each of the chimeric receptor constructs. Accordingly, a direct comparison of its effects on each receptor type was performed. The Gd^{3+} concentration-response characteristics of the Ca^{2+} receptor, CaR/mGluR1, mGluR1/CaR, and Δ ntCaR are compared in Fig. 6. These results show that the Ca^{2+} receptor and CaR/mGluR1 both respond to Gd^{3+} with nearly identical EC_{50} values. The Δ ntCaR was also responsive to Gd^{3+} , although higher concentrations of ligand were necessary for its activation than for the native Ca^{2+} receptor or the CaR/mGluR1 chimera. Curiously,

the EC_{50} for Gd^{3+} activation of the mGluR1/CaR was even lower than for activation of the native Ca^{2+} receptor.

Cooperativity of Activation. Activation of the Ca^{2+} receptor by cationic ligands is characterized by a relatively steep concentration-response relationship. The cation concentration-response curves to polyvalent cations obtained in this study are consistent with previously reported studies of Ca^{2+} receptor activation in parathyroid cells or after its expression in *X. laevis* oocytes (Nemeth and Scarpa, 1986; Brown, 1991; Brown et al., 1993; Garrett et al., 1995). As expected, glutamate activation of the mGluR1 expressed in oocytes followed the more typical concentration-response curve to yield a Hill coefficient (n_H) of slightly greater than one. The steepness of the cation dose-response relationship was maintained for both the CaR/mGluR1 and the mGluR1/CaR ($n_H > 3$). Using a selected group of known agonists, complete activation of the mGluR1/CaR by Gd^{3+} occurs within a 10-fold concentration range, whereas activation by mGluR1 agonists occurs over approximately a 100-fold agonist concentration range in both the mGluR1 and mGluR1/CaR (Fig. 7). It seems that cation activation, whether mediated by sites within the ECD or TMD, exhibits strong cooperativity.

Discussion

The functional characterization of Ca^{2+} receptor-mGluR1 chimeras in this study demonstrates that, although these two receptor types share limited sequence homology (about

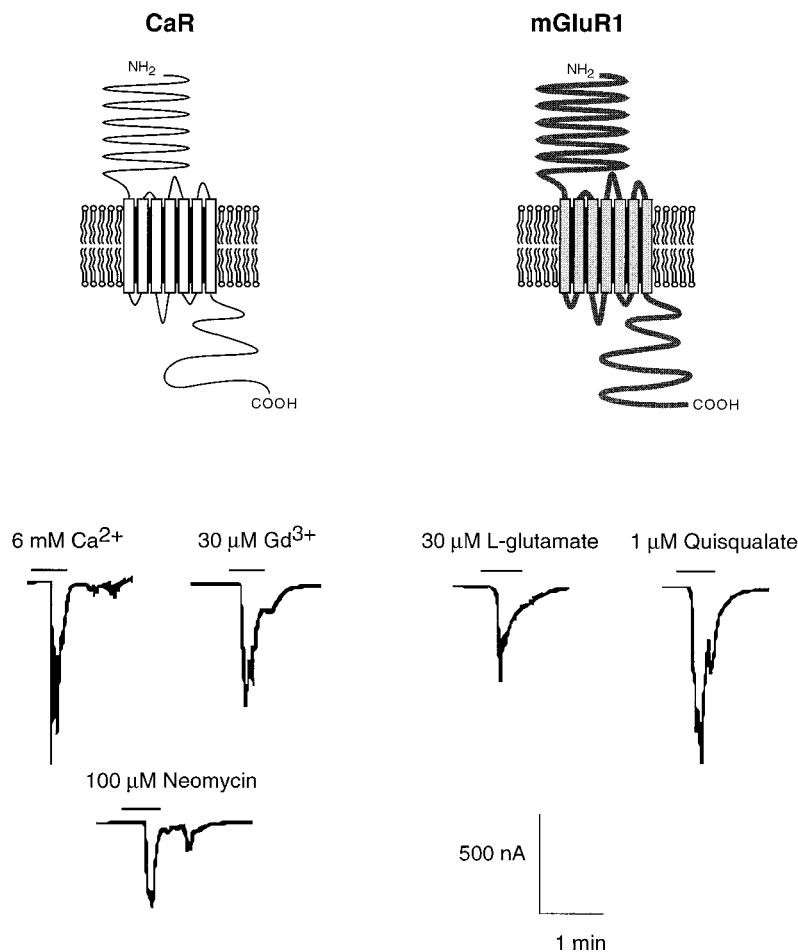


Fig. 1. Agonist activation of the Ca^{2+} receptor and the mGluR1a by their respective agonists. Currents were recorded from oocytes voltage-clamped at -60 mV and agonists were applied by superfusion for durations indicated by the horizontal bars above each trace.

25%), their overall structural homology is sufficient to enable functional complementation. The similarities in overall topology and the clear differences in agonist pharmacology between the two receptor types have provided an approach for the determination of sequence regions that are responsible for various aspects of receptor function, including agonist recognition and receptor activation. Thus, it seems that, like the mGluRs, ligand binding and receptor activation of the Ca²⁺ receptor are primarily determined by the ECD and the serpentine TMD, respectively.

Takahashi et al. (1993) reported that the large ECD of mGluRs played a dominant role in determining mGluR agonist rank order of potency. They constructed several chimeric receptors by interchanging segments within the ECDs of the mGluR1 and mGluR2 subtypes and found that the differences in agonist selectivity between the two subtypes were determined by sequences within the ECD. Parmentier et al. (1998) further demonstrated that ligand recognition of mGluRs is confined to the ECD. The present study supports

and extends these earlier findings by providing evidence that the amino-terminal ECD, exclusive of the TMD, contains functional domain(s) for agonist responsivity. The Ca²⁺ receptor does not respond to mGluR agonists, but the mGluR1/CaR chimera is activated by glutamate and quisqualate at concentrations very similar to those necessary for activation of the native mGluR1 expressed in oocytes. This functional complementation suggests that the binding of an agonist to the ECD of one receptor type induces conformational changes in the TMD that produce a functional response.

Data obtained from analysis of the CaR/mGluR1 are most convincing in terms of the role of the Ca²⁺ receptor ECD in cation recognition. The cation agonist profile of the CaR/mGluR1 is nearly indistinguishable from that of the native Ca²⁺ receptor. Ca²⁺, Gd³⁺, and neomycin all activate the CaR/mGluR1 and their EC₅₀ values do not differ significantly from those obtained with the native Ca²⁺ receptor. Glutamate and quisqualate are ineffective, even at concentrations as high as 1 mM. These results alone indicate that the ECD of the Ca²⁺ receptor contains necessary and sufficient sites for agonist recognition, particularly by the endogenous ligand, Ca²⁺.

The ability of certain cations to activate the mGluR1/CaR suggests that the TMD of the Ca²⁺ receptor contains cation recognition sites that are capable of receptor activation. Curiously, Gd³⁺ is not only the most potent Ca²⁺ receptor agonist of the mGluR1/CaR, it is also a more potent agonist of the mGluR1/CaR than of the native Ca²⁺ receptor. Ca²⁺ and neomycin, however, essentially lack activity at mGluR1/CaR. The reason for these differences is not clear. Although expression levels were not determined for the native and chimeric receptors, there is evidence to suggest that differences in agonist selectivity are not caused simply by variations in expression level. For example, activation by Gd³⁺ of either the CaR/mGluR1 or the mGluR1/CaR produces similar increases in chloride current amplitudes, but these receptors differ significantly in their ability to respond to calcium or neomycin.

When the mGluR1 is expressed at very high levels, as

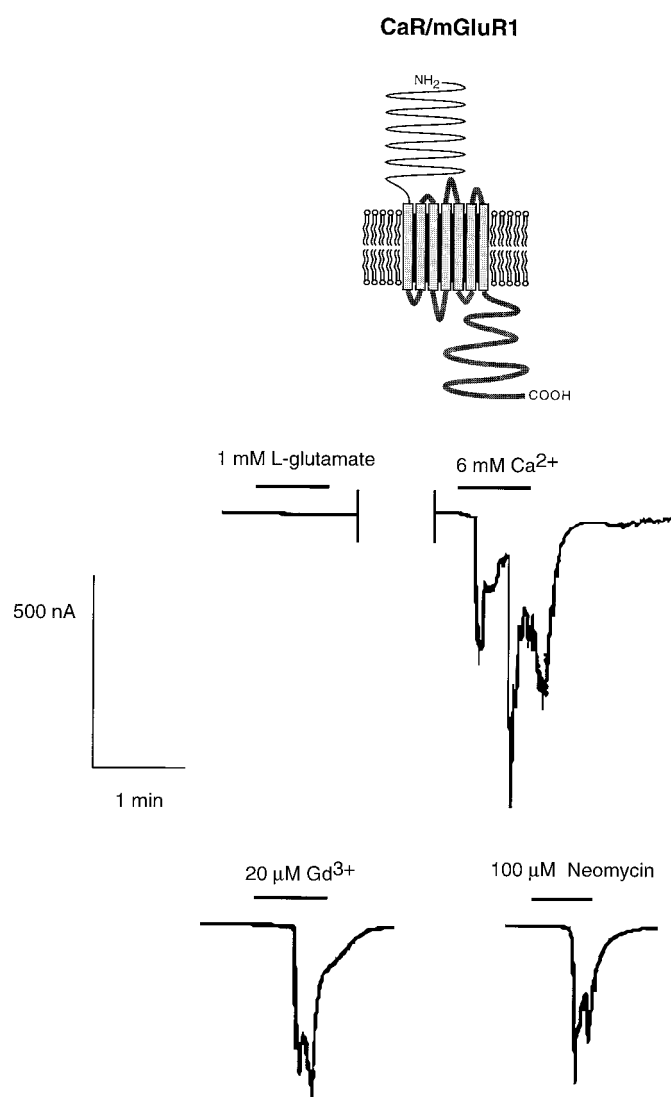


Fig. 2. Agonist activation of the CaR/mGluR1 chimera. The effects of L-glutamate and cation agonists of the CaR were examined in oocytes injected with cRNA encoding the CaR/mGluR1. The duration of agonist application is indicated by the horizontal bars above the current trace. The break in the trace represents approximately 5 min.

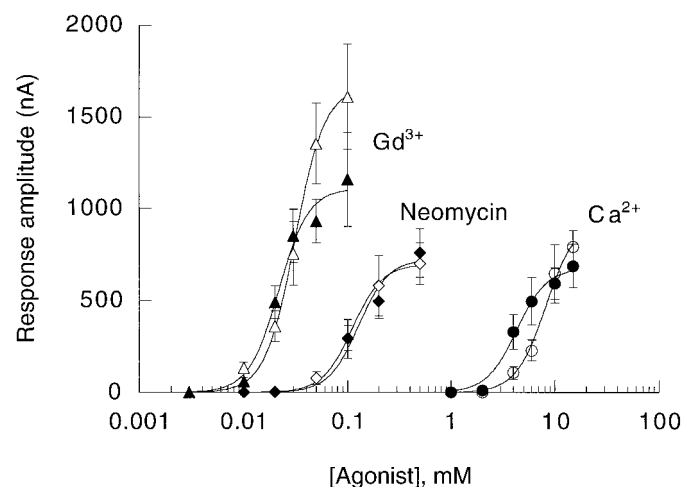


Fig. 3. Concentration-response analysis of cation activation of the CaR/mGluR1 and the native CaR. Data points represent the mean \pm S.E.M. for 4 to 17 oocytes in each determination. The EC₅₀ values obtained for Ca²⁺, Gd³⁺ and neomycin on the native Ca²⁺ receptor were 8.1 \pm 0.5 mM, 21 \pm 1.5 μ M, and 127 \pm 16 μ M, respectively, and on the CaR/mGluR1, 4.7 \pm 0.3 mM, 31 \pm 10 μ M, and 112 \pm 29 μ M, respectively.

a

mGluR1/CaR

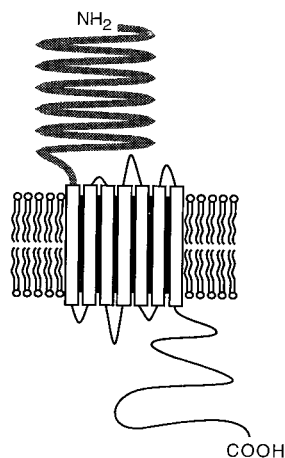
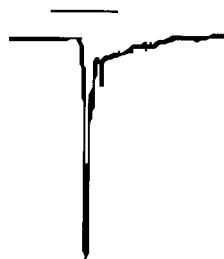
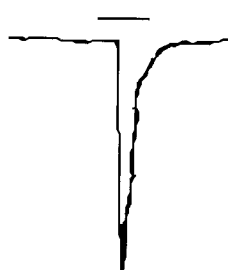
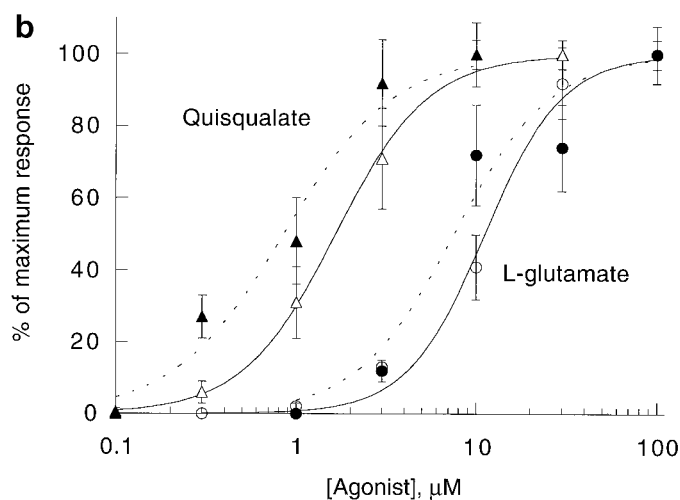
30 μM L-glutamate1 μM Quisqualate3 μM Gd^{3+} 

Fig. 4. Agonist activation of the mGluR1/CaR. A, representative traces showing responses to the application of glutamate receptor agonists and Gd^{3+} . B, concentration-response analysis of glutamate agonist activation of the mGluR1 and the mGluR1/CaR chimera. Data points represent the mean \pm S.E.M. for 3 to 15 oocytes for each determination. The EC_{50} values obtained for L-glutamate and quisqualate in the native mGluR1 were $17.2 \pm 0.7 \mu\text{M}$ and $1.7 \pm 0.1 \mu\text{M}$, respectively. The EC_{50} values obtained for L-glutamate and quisqualate on the mGluR1/CaR were 11.9 ± 0.8 and 1.1 ± 0.2 , respectively.

500 nA

1 min



suggested by unusually large responses to glutamate agonists, responses to Gd^{3+} (at concentrations of at least $10\ \mu\text{M}$) are observed, as are spontaneous oscillations in the absence of added quisqualate or glutamate. The spontaneous activity is most likely explained as a result of an active receptor/G protein conformation in the absence of agonist. The responses to Gd^{3+} by the mGluR1/CaR and the ΔntCaR differed from the responses to Gd^{3+} by mGluR1a in that the mGluR1a responses are not consistent from oocyte to oocyte and from frog donor to frog donor under our experimental conditions. A possible explanation for these Gd^{3+} responses may be that the mGluRs and the Ca^{2+} receptor share a common ancestral receptor and that the apparent cation-sensing properties of the mGluR1 may be caused by the presence of a vestigial cation recognition site. Recent mutagenesis of mGluRs showed that a specific serine residue found in certain mGluRs is responsible for their apparent cation sensing (Kubo et al., 1998). However, rat cortical astrocytes express mGluR5, yet mGluR5 activation by selective mGluR agonists is unaffected by Gd^{3+} in this system (M. Logan and L. Hammerland, unpublished observations). Ad-

ditionally, the Gd^{3+} effect may result from subtle conformational changes that enhance the active receptor/G protein complex. Cations are known to modulate the activity of many GPCRs (Neve, 1991; Ceresa and Limbird, 1994); whether this effect of Gd^{3+} is related to those previously described effects of cations on the function of other GPCRs is not clear.

The agonist pharmacological profiles of the ΔntCaR and the mGluR1/CaR suggest that the TMD contains cation agonist interaction sites. Both receptors respond well to Gd^{3+} , but the activities of Ca^{2+} and neomycin are greatly reduced in the mGluR1/CaR and are nearly undetectable in the ΔntCaR . These data, together with the observation that cation responsiveness of the CaR/mGluR1 chimera and the Ca^{2+} receptor are nearly identical, provide further evidence that the primary determinants for cation activation of the CaR are found within the ECD. Furthermore, it seems that any other site within the TMD may not be necessary for activation by the natural ligand.

The cooperativity of agonist activation is maintained with respect to cation versus glutamate agonist activation. For

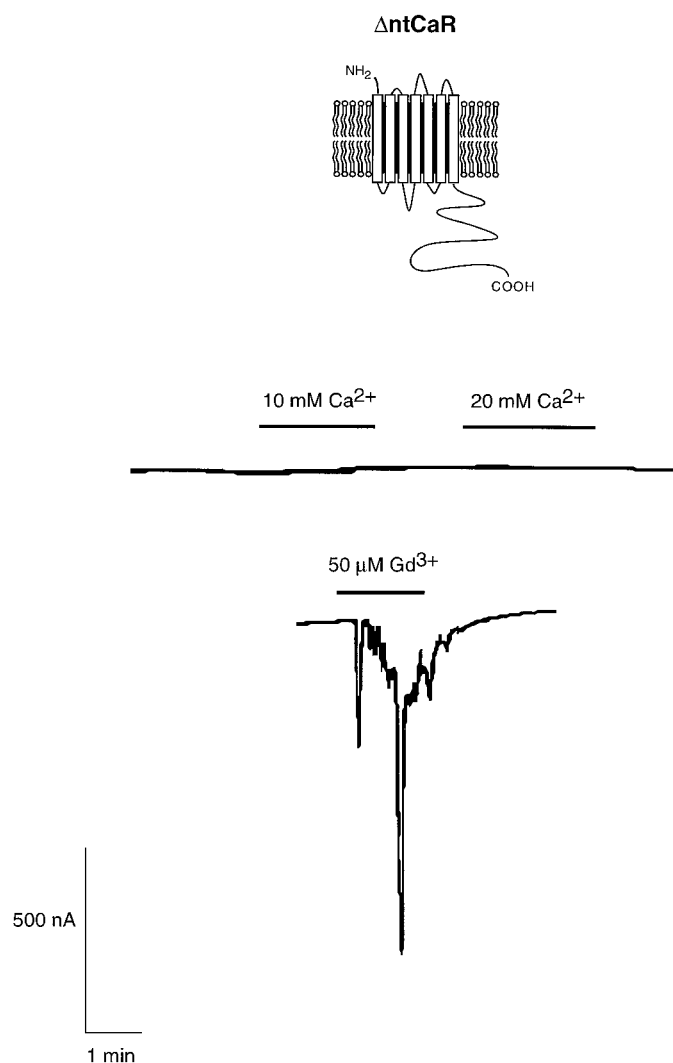


Fig. 5. Gd^{3+} activation of the ΔntCaR . Oocytes were injected with cRNA encoding the ΔntCaR 4 days before assay and agonists were applied by superfusion for the times indicated by the horizontal bars above the traces.

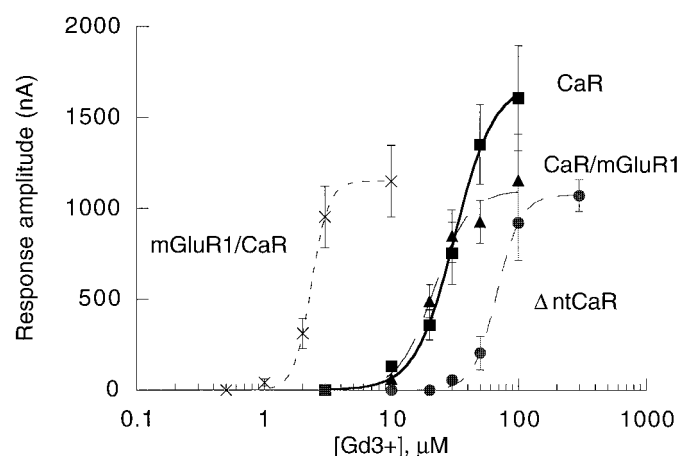


Fig. 6. Concentration-response analysis of Gd^{3+} activation of the CaR, CaR/mGluR1, mGluR1/CaR and ΔntCaR . Data points represent the mean \pm S.E.M. for 4 to 17 oocytes for each determination. The EC_{50} values obtained for Gd^{3+} on the mGluR1/CaR and the ΔntCaR were $2.3 \pm 0.4\ \mu\text{M}$ and $67.9 \pm 14\ \mu\text{M}$, respectively.

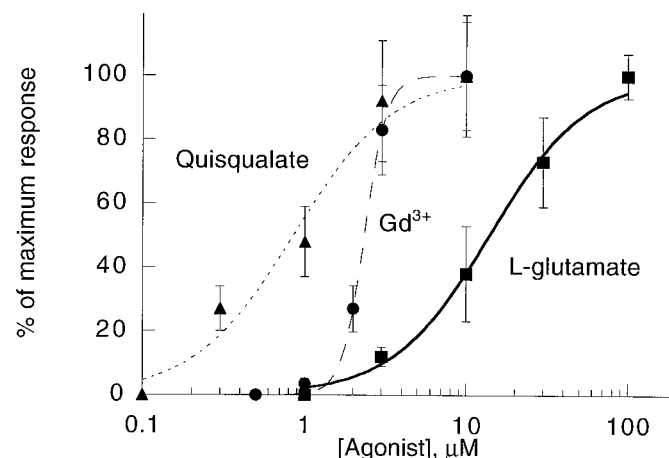


Fig. 7. Cooperativity of mGluR1/CaR activation by glutamate agonists and Gd^{3+} . The agonist activation kinetics of the mGluR1/CaR in response to L-glutamate, quisqualate and Gd^{3+} . Data points represent the mean \pm S.E.M. for 3 to 17 oocytes for each determination. The n determined for quisqualate activation was 1.4 ± 0.32 and for L-glutamate it was 1.67 ± 0.57 , whereas the n for activation by Gd^{3+} was 5.2 ± 1.2 .

example, activation of the native mGluR1 or the mGluR1/CaR chimera by glutamate or quisqualate is characterized by concentration-response curves that extend to nearly two log units of agonist concentrations. Conversely, activation of the native CaR, CaR/mGluR1, mGluR1/CaR, or Δ ntCaR by cations, regardless of whether the recognition site is found within the ECD or TMD, results in a dramatically steeper concentration-response relationship.

The current understanding of the structure/function relationships of GPCRs, with respect to agonist recognition and activation, includes at least three primary patterns (for review, see Coughlin, 1994). For receptors that bind small molecule ligands, such as the adrenergic and muscarinic receptors, these ligands bind to pockets within the TMD (Kobilka et al., 1988; Strader et al., 1989; Dohlman et al., 1991; Strader et al., 1991). Members of the chemokine and glycoprotein receptor families seem to contain sites for agonist recognition in both the ECD and TMD (Moyle et al., 1991; Nagayama et al., 1991; Fong et al., 1992a, 1992b; Buggy et al., 1995). To date, however, the mGluRs seem to be distinct in that the sites of agonist interaction are found exclusively in the ECD (O'Hara et al., 1993; Takahashi et al., 1993). The data presented in this report also support that suggestion regarding the mGluRs.

The activity of cation agonists of the Ca^{2+} receptor on the CaR/mGluR1 chimera suggests that, much like the mGluRs, the ECD of the Ca^{2+} receptor contains the binding site(s) for the physiological ligand (i.e., extracellular Ca^{2+}). The large ECD of each receptor confers sensitivity to agonist activation. However, unlike the mGluRs, the TMD of the CaR may also contain determinants for some cation interaction. These results provide a basis for even more refined approaches to characterizing ligand binding sites.

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