Amino Acids in the Second and Third Intracellular Loops of the Parathyroid Ca\textsuperscript{2+}-sensing Receptor Mediate Efficient Coupling to Phospholipase C*

To determine the role of amino acids in the second and third intracellular (IC) loops of the Ca\textsuperscript{2+}-sensing receptor (CaR) in phospholipase C (PLC) activation, we mutated residues in these loops either singly or in tandem to Ala and assessed PLC activity by measuring high extracellular \([\text{Ca}^{2+}]_\text{o}\) (\([\text{Ca}^{2+}]_\text{i}\))-induced inositol phosphate accumulation and protein expression by immunoblotting and immunocytochemistry in human embryonic kidney 293 cells. Two CaR constructs in the second IC loop, F707A CaR and to a lesser extent L704A CaR, demonstrated reduced activation of PLC, despite levels of protein expression comparable with the wild-type (wt) CaR. Substitution of Tyr or His for Phe-707, but not Leu, Val, Glu, or Trp, partially restored the ability of high \([\text{Ca}^{2+}]_\text{i}\) to activate PLC. Eight residues in the third IC loop were involved in PLC signaling. The responses to high \([\text{Ca}^{2+}]_\text{i}\) in cells expressing CaRs with Ala substitutions at these sites were <35% of the wt CaR. The L798A, F802A, and E804A CaRs were dramatically impaired in their responses to \([\text{Ca}^{2+}]_\text{i}\), even up to 30 mm. Substitutions of Leu-798 with other hydrophobic residues (Ile, Val, or Phe), but not with acidic, basic, or polar residues, produced reduced responses compared with wt. Phe-802 could be replaced with either Tyr or Trp with partial retention of the ability to activate PLC. Glu-804 could only be substituted with Asp or Gln and maintain its signaling capacity. Cell surface expression of the CaRs mutated at Leu-798 and Phe-802 appeared normal compared with wt CaR. Cell surface CaR expression was, however, reduced substantially in cells expressing several mutants at position Glu-804 by confocal microscopy. These studies strongly implicate specific hydrophobic and acidic residues in the second and third IC loops of the parathyroid CaR (and potentially larger stretches of the third loop) in mediating efficient high \([\text{Ca}^{2+}]_\text{i}\)-induced PLC activation and or CaR expression.

CaRs are members of the G-protein-coupled receptor (GPCR) superfamily and couple to PLC activation, inhibition of cyclic AMP formation, and opening of nonselective cation channels (1–4). CaRs share modest sequence homology with the metabotropic glutamate receptors (mGluRs) (5, 6), the type B \(\gamma\)-aminobutyric acid receptor (GABA\(_\text{B}\)) (7), and a large group of pheromone receptors (8, 9) and, thus, constitute the family 3 of GPCRs (10).

Receptors in the CaR/mGluR subfamily share several structural features. These include a large extracellular amino-terminal domain, seven membrane-spanning regions, three IC loops, and a large cytoplasmic tail (see Fig. 1a). The extracellular domains of CaRs and mGluRs are known to be critical for ligand recognition (11–13). Naturally occurring mutants of the CaR, implicated in the pathogenesis of abnormal Ca\textsuperscript{2+}-sensing in vivo, occur predominantly in the large amino-terminal domain of this receptor (14, 15). Point mutations in this domain, responsible for either gain-of-function or loss-of-function, indicate its key role in the Ca\textsuperscript{2+}-sensing function of the receptor.

IC domains of receptors in the CaR/mGluR subfamily are likely, by analogy to other GPCRs, to be responsible for coupling to G-protein-mediated signal transduction (10, 16, 17). A comparison of CaRs with the mGluR 1–8 indicates limited sequence conservation in their second IC loops (<10%) but striking conservation (67 to 85%) in their third IC loops (see Fig. 1b). This observation suggests these latter regions likely share similar function.

Mutagenesis of mGluR1 and R5 previously demonstrated that specific residues in IC loops 2 and 3 contribute to PLC activation, whereas other residues were involved in the regulation of cyclic AMP formation (5). Domains of comparable functional significance in the CaR have, to date, not been identified. Studies of kindred with familial benign hypercalcemia and neonatal severe hyperparathyroidism indicated that a CaR with a mutation at residue 795 (R795W) in the amino-terminal portion of the third IC loop had a reduced ability to mobilize intracellular Ca\textsuperscript{2+} (18). The remaining residues within the second and third IC loops have not been carefully examined. In these studies, we mutated amino acids in IC loops 2 and 3 of the bovine CaR to identify the positions of key signaling residues and structural requirements at those sites. Phe-707 in the second IC loop and 2 residues in the third IC loop, Leu-798 and Phe-802, proved critical to the activation of PLC. Glu-804 proved essential for efficient cell surface expression of CaR. This work supports the presence of several functional determinants in IC loops 2 and 3 in the stimulation of PLC by and expression of CaRs in mammalian cells.
EXPERIMENTAL PROCEDURES

Materials—The full-length bovine parathyroid CaR cDNA (1) was provided by Dr. Edward Brown (Harvard Medical School, Boston, MA). The Chameleon double-stranded, site-directed mutagenesis kit and pBluescript II SK- (pBS) were obtained from Stratagene (La Jolla, CA). pcDNA1/Amp and pCEP4 were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes were from Stratagene, Life Technologies, Inc., and Promega (Madison, WI). Fluorescein-conjugated goat-anti rabbit IgG for immunocytochemistry was obtained from Molecular Probes, Inc. (Eugene, OR). Other supplies were from previously noted sources (3, 19).

Mutagenesis and Subcloning of wt and Mutant CaR cDNAs—The SmaI fragment (nucleotides 248–3819) of the wt bovine parathyroid CaR was ligated into pBS (wt CaR/pBS) as described previously (3) and used as the template for mutagenesis. Mutagenesis was performed using the Chameleon kit according to the manufacturer’s instructions. Briefly, in each reaction a selection primer was used to convert a unique KpnI site in wt CaR/pBS to an SrfI site, and a mutagenic primer was used to introduce the desired mutation and a new restriction site (i.e. NotI, NspV, SphI, HindIII, SmaI, Nar I, or DraI). Primers were mixed and annealed with heat-denatured plasmid cDNA template. Synthesis of the (−) strand of plasmid cDNA was achieved by DNA polymerase and ligase. The reaction mixture was then treated with KpnI to linearize any double-stranded wt CaR cDNA. Uncut circular hybrid cDNA was then transformed into XL-mutS blue E. coli. The latter construct was made by ligating the 6308-bp XhoI fragment of wt CaR/pcDNA1/Amp and annealed with heat-denatured plasmid cDNA template. Synthesis of the (+) strand of plasmid cDNA was achieved by DNA polymerase and ligase. The reaction mixture was then treated with KpnI to linearize any double-stranded wt CaR cDNA. Uncut circular hybrid cDNA was then transformed into XL-1 blue E. coli. Transformants were selected on Luria-Bertani broth agar plates containing ampicillin. After DNA amplification, mutations were confirmed by automated DNA sequencing (Biomolecular Resource Center, University of California, San Francisco).

Subcloning of mutant CaR constructs into pcDNA1/Amp for transient transfections were done by gel-purifying the 2213-bp PinAI-XbaI fragment, which contained the mutations in the second IC loop, and ligating this into the 6308-bp PinAI-XbaI fragment of wt CaR/pcDNA1/Amp. The latter construct was made by ligating the 3619-bp fragment of wt CaR/pBS into pcDNA1/Amp cut with NotI and HindIII. For constructs with mutations in IC loop 3, a 1073-bp XhoI-XbaI fragment from the relevant pBS mutant CaR construct was gel-purified and ligated into the 7448-bp XhoI-XbaI fragment of wt CaR/pcDNA1/Amp. To generate mutant CaR constructs for stable transfections, the SrfI site in mutant CaR/pBS constructs was first converted back to KpnI site by mutagenesis. The KpnI-NotI fragment comprising the mutant CaR cDNA of interest was then subcloned into pCEP4 as described (3).

Transient and Stable Transfections—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium with fetal bovine serum (10% v/v) and transfected as described previously using CaCl2 precipitation (3). For transient expression, cells were washed twice with phosphate-buffered saline after a 24-h incubation and then plated in 6-well culture plates. After allowing 48 to 72 h for attachment and growth, InsP production and receptor expression were assessed. For stable expression of CaR constructs, transfected cells were selected in medium containing hygromycin B (200 μg/ml) for at least 4 weeks before experiments.

InsP Assay—Total InsP accumulation was measured in triplicate after labeling transfected HEK293 cells with [3H]myo-inositol (2 μCi/ml) for 18 to 24 h in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum as described (3). [3H]InsP accumulation in the presence of LiCl (10 mM) was measured after a 60-min incubation with different [Ca2+]o at 37 °C. Total [3H]InsPs were extracted from cells and isolated by anion-exchange chromatography (3, 20). Results in all experiments are reported as the average fold-increase in total [3H]InsP and calculated as total [3H]InsPs produced by increasing [Ca2+]o/μmol [3H]InsPs at 0.5 mM Ca2+.

Immunoblotting and Immunocytochemistry—Crude membrane protein fractions were prepared from HEK293 cells, electrophoresed on SDS-polyacrylamide electrophoresis gels, and transferred to nitrocellulose membranes as described (3, 19). Membranes were blotted with one of two affinity-purified rabbit antisera (21825B, 50 nM or 321113A, 10 mM) (3, 19), and signals were detected with an enhanced chemiluminescence (ECL™) assay kit (Amersham Pharmacia Biotech). Protein expression of all mutant CaR constructs was tested by immunoblotting at least twice along with wt CaR controls. Immunocytochemistry with 3,3′-diaminobenzidine- and fluorescein-conjugated antibodies was performed on cells grown on chamber slides, fixed for 30 min, incubated with primary and secondary antibodies, and then stained as previously detailed (3, 19). Primary antibody was either a CaR antiserum (21825A; 500 μg/ml), this antiserum preincubated with 100-fold excess peptide, or non-immune rabbit serum. The antiserum was raised against a peptide derived from the extracellular domain of the bovine parathyroid CaR (3, 19). For 3,3′-diaminobenzidine-staining, slides were counterstained with aqueous hematoxylin. For fluorescein staining, coverslips with cells were mounted on glass slides using Gel-Mount (Biomeda, Foster City, CA) without counterstaining and examined with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany). All mutants were analyzed from at least two separate transfections along with wt CaR-expressing cells. Coverslips were coded and then examined by two blinded observers, who assessed staining patterns without knowledge of the mutation status.

Statistics—Differences between wt and mutant CaR responses were tested by analysis of variance with the F-test using Excel 98 (Microsoft, Seattle, WA).

RESULTS

Signaling Properties and Expression of Mutants in the Second IC Loop of CaR

Transient Transfections—To assess the importance of residues in the second IC loop of the bovine parathyroid CaR to signal transduction, we constructed seven mutants in which four sequential amino acids were converted to Ala (tandem Ala...
antiserum confirmed that the sizes, intensity, and patterns of the protein bands detected in cells expressing all seven TA mutant CaR constructs were comparable with those of wt CaR-expressing HEK293 cells (Fig. 2b and data not shown). Protein bands in membranes from both wt and mutant CaR-expressing cells were ~140 and 160 kDa and of equivalent intensity. Thus, differences in the relative quantity or forms of receptor proteins expressed do not explain the reduced signaling properties of these two mutants.

To identify specific signaling determinants within the amino-terminal part of the second IC loop, we mutated residues 700–707 individually to Ala and assessed the ability of these point mutants to support InsP production. Cells transiently transfected with L705A and V706A CaR cDNAs responded to raising [Ca\(^{2+}\)]\(_o\) from 0.5 to 5.0 mM with 9.5–10-fold increases in InsPs, comparable with the responses of the wt CaR (10.7 ± 0.3-fold) in these experiments. Cells transiently expressing the mutants T700A, N701A, R702A, and V703A CaRs had mildly reduced InsP responses to the same increment in [Ca\(^{2+}\)]\(_o\) of 6–9-fold. These were ~15 to 40% lower than the wt CaR (Fig. 3a). The most dramatic defects, however, were observed in cells expressing L704A and F707A CaR mutants. A 4.5 mM increase in [Ca\(^{2+}\)]\(_o\) produced increases in InsPs of only 2.9 ± 0.4 (L704A CaR; p < 0.01 versus wt) and 0.2 ± 0.1-fold (F707A CaR; p < 0.001 versus wt), which is 27 and 2% of wt CaR responses, respectively (Fig. 3a).

Dose-response studies showed that the L704A CaR mutant had an ED\(_{50}\) for Ca\(^{2+}\) that was right-shifted to 7.3 ± 0.09 mM Ca\(^{2+}\) (p < 0.01, compared with ~5.5 ± 0.2 mM Ca\(^{2+}\) for wt CaR; see Fig. 3b). This mutant and the wt CaR produced comparable maximal InsP responses (\(R_{max}\)) of 6.8 ± 1.1 and 7.3 ± 1.1-fold increases at 30 mM Ca\(^{2+}\), respectively. In contrast, the F707A CaR mutant generated an \(R_{max}\) of only a 0.91 ± 0.3-fold increase over basal (p < 0.001 versus wt) (Fig. 3b). Immunoblotting and immunocytochemistry confirmed comparable levels of receptor expression for the eight single-site mutants including L704A and F707A CaRs in HEK293 cells (Fig. 3c and data not shown).

**Stable Transfections**—To confirm that the signaling defects of the IC loop 2 mutants were not related to transient expression, we stably transfected wt and mutant L704A and F707A CaRs into HEK293 cells. Responses of the wt CaR-expressing cells to [Ca\(^{2+}\)]\(_o\) were much greater in stably versus transiently transfected cells, as previously reported (3), due to higher levels of receptor expression. In cells stably expressing wt CaRs, increasing [Ca\(^{2+}\)]\(_o\) from 0.5 to 5.0, 10, or 30 mM increased InsPs by 36.5 ± 2.7-, 47.0 ± 4.6-, or 36.7 ± 5.4-fold, respectively (Fig. 3d). Experiments with cells stably expressing L704A and F707A CaRs confirmed the severe reduction in InsP responses to high [Ca\(^{2+}\)]\(_o\) (see Fig. 3d), similar to the results from transient transfections. The ED\(_{50}\) was significantly right-shifted from 3.3 ± 0.1 (wt CaR) to 5.1 ± 0.2 mM Ca\(^{2+}\) (L704A CaR) (p < 0.03) (Fig. 3d), similar to the shift observed in transient transfections (Fig. 3b). The \(R_{max}\) at 30 mM Ca\(^{2+}\) of the L704A CaR was also significantly reduced to a 21.2 ± 5.6-fold-increase compared with that of the wt CaR, which yielded 47.0 ± 4.5-fold increases in InsPs in stably transfected cells (p < 0.01) (Fig. 3d). In contrast, we had observed no substantial difference between maximal signaling (at 30 mM Ca\(^{2+}\)) of the L704A CaR and the wt CaR in transient transfections (Fig. 3b). The F707A CaR, however, remained severely impaired in its ability to activate PLC in stably transfected cells. Its ED\(_{50}\) was 6.6 ± 0.2 mM Ca\(^{2+}\), which was significantly greater (p < 0.01) than that of the wt CaR (3.3 ± 0.1 mM Ca\(^{2+}\)). The \(R_{max}\) of this mutant averaged a 7.3 ± 0.6-fold increase at 30 mM Ca\(^{2+}\), which was only 16% of wt CaR responses (p < 0.001) (Fig. 3d).
Both L704A and F707A CaRs were expressed at levels comparable with wt CaR by immunoblotting and immunocytochemistry (Fig. 3c and data not shown). Overall, these findings suggest that the signaling defects seen with L704A or F707A CaR mutants were not due to substantial reductions in receptor expression or alterations in receptor processing. These results support the idea that Leu-704 plays a secondary role, whereas Phe-707 is absolutely essential in PLC-mediated signal transduction by the CaR.

**Role of Phe-707 in CaR-induced Activation of PLC**

To test whether the phenyl side chain of Phe-707 is essential for activation of PLC, we mutated this residue to others with functional groups of different sizes and charges (e.g. Val, Leu, Glu, His, Tyr, and Trp). Substitution of Phe-707 with hydrophobic residues Val or Leu produced CaR mutants that did not respond to raising [Ca\(^{2+}\)], from 0.5 to 5.0 mM (Fig. 3f). HEK293 cells transiently expressing F707A CaRs, in which Phe was converted to a negatively charged amino acid, were also unresponsive to raising [Ca\(^{2+}\)] to 5.0 mM (Fig. 3f). Substitution of Phe-707 with negatively charged His yielded a CaR mutant whose response to 5 mM Ca\(^{2+}\) was reduced by ~75% compared with the wt receptor (p < 0.01) (Fig. 3f). Substitution of Phe-707 with Trp, an even bulkier side group than Phe, generated a receptor whose ability to activate PLC (Fig. 3f). Substitution of Phe-707 with Tyr produced a receptor whose ability to increase InsPs was ~50% that of wt responses (p < 0.05) (Fig. 3f). The CaRs mutated at position 707 that we studied were expressed at levels comparable with the wt CaR by immunoblotting (data not shown). These results suggested that there was relatively little tolerance for changes of the nonpolar aromatic side chain of Phe-707 in mediating CaR signaling through PLC. Failure of the Trp substitution to maintain CaR function further suggested that an aromatic side chain larger than Phe also disrupted the function that this residue serves in the CaR.

**Mutational Analysis of the Third IC Loop of the CaR**

To assess contributions of the third IC loop of the CaR in signal transduction, we next individually mutated amino acids 794–807 to Ala (except 805, which is an Ala in the wt sequence) and assessed the ability of these mutants to activate PLC in HEK293 cells (Fig. 1). Mutations of 11 residues in this region produced CaRs with altered signaling responses, which we divided into two groups (Fig. 4a). Group 1 mutants, including K794A, N801A, and F807A CaRs, were able to increase InsPs with raising [Ca\(^{2+}\)] from 0.5 to 5.0 mM by ~6.8–7.7-fold. Their responses, however, were ~55–63% that of the wt CaR (12.2±1.1-fold) and were statistically significantly reduced (p < 0.01 versus wt; Fig. 4a). Group 2 mutants, comprising the eight remaining CaR constructs, were more impaired than group 1 mutants; their InsP responses to the same increment in [Ca\(^{2+}\)] were <35% that of the wt CaR (p < 0.003; see asterisks in Fig. 4a).
Ca2+ Receptor Activation of PLC

Reduced Sensitivity to [Ca2+]o—The $R_{\text{max}}$ of the N803A CaR was a 14.4 ± 6.0-fold increase in InsPs at 30 mM Ca2+, which was comparable with the wt CaR (15.2 ± 1.2-fold). The ED50 of this mutant (~7.5 mM Ca2+) was, however, modestly shifted to the right compared with wt CaR (~3.5 mM Ca2+) (Fig. 4b).

Reduced Sensitivity and Reduced Maximal Responses to [Ca2+]o—Four mutants exhibited both reduced $R_{\text{max}}$ and increased ED50 values (i.e. R796A, K797A, P799A, and K806A CaRs). Their ED50 values ranged from 6 to 8 mM Ca2+, and their responses to 30 mM Ca2+ were only ~36–60% that of the wt CaR responses ($p < 0.01$; Fig. 4c).

Signaling-defective CaRs—Three mutant receptors (L798A, F802A, and E804A CaRs) were unable to activate PLC even at 30 mM Ca2+. Their responses to high [Ca2+]o were only 1.2–1.8-fold above basal (at 0.5 mM Ca2+) and were equivalent to the increases in the vector controls at 30 mM Ca2+ (1.4 ± 0.2-fold; Fig. 4b).

To address whether any of the above signaling abnormalities could be attributed to defective receptor expression, we analyzed CaR expression by Western blotting and immunocytochemistry. The levels and patterns of CaR protein bands for both wt and mutant CaRs were similar (Fig. 4d). Immunocytochemistry of cells expressing the Ala mutants was performed and showed that cell surface expression in all but one mutant (E804A CaR) was comparable with the wt CaR (see Fig. 5). Cells expressing this mutant had less receptor staining on the membrane and increased staining in intracellular organelles (Fig. 5: wt versus E804A). These observations supported the idea that the marked decreases in PLC activation observed with the Ala mutants of the third IC loop of CaR (except for E804A) were likely due to defective receptor-effector signaling and not due to reduced expression of the receptor. Since E804A CaRs were so aberrant in their expression pattern, their ability/ inability to mediate PLC activation could not be tested.

Mutagenesis of Leu-798, Phe-802, and Glu-804

The three sites identified above (798, 802, and 804) were then further mutagenized to address the amino acid requirements at these positions to support PLC activation and efficient receptor expression.

Leu-798—The observation that a CaR mutant with Ala substituted for Leu-798 did not activate PLC equivalently to the wt CaR led us to hypothesize that the size of the hydrophobic residue at this position was critical for signal transduction. To test this hypothesis, we substituted Leu-798 with amino acids with different types of side chains. As shown in Fig. 6a, only the substitution of Leu-798 with very closely related Ile produced a receptor that could increase InsPs at 30 mM Ca2+ to levels comparable with the wt CaR. The ED50 for the L798I CaR was modestly shifted to the right from 5 to 7.5 mM Ca2+.

All other substitutions for Leu-798 (namely Val, Phe, Glu, Pro, and Lys) produced CaRs that were marked defective (i.e. reduced by > 75%) in their ability to activate PLC, even with [Ca2+]o, as high as 30 mM (Fig. 6a and b). The substitution of a basic (Lys) or acidic (Glu) residue for Leu-798 was poorly tolerated, as was the nonpolar rigid side chain of Pro. High
[Ca\textsuperscript{2+}]_i-induced InsP responses of cells expressing these three mutant CaRs were equivalent to vector controls (Fig. 6b).

Taken together, these findings underscored the importance of a specific, nonpolar hydrocarbon side chain at position 798 in the ability of CaRs to mediate PLC activation.

**Phe-802**—To examine the role of the side chain at position 802 in the CaR in PLC activation, we mutated this Phe to Val, Leu, Glu, His, Tyr, and Trp. As expected, closely related Tyr was essentially interchangeable with Phe. The F802Y CaR mutant increased InsPs similarly to the wt CaR (Fig. 7a).

Substitution of Trp, a bulkier residue than Phe, produced a mutant with an R\textsubscript{max} of 60% that of wt controls (Fig. 7a). There were no significant changes in the ED\textsubscript{50} for Ca\textsuperscript{2+} with either the F802Y or F802W CaR. The responses of the other four mutants (Phe-802 converted to His, Leu, Glu, and Val) were markedly reduced compared with wt CaR responses (p < 0.001) and indistinguishable from vector controls (see Fig. 7, a and b). These results suggested that the aromatic side chain of Phe at position 802 in the CaR in PLC activation, we mutated this Phe to Val, Leu, Glu, His, Tyr, and Trp.

**Glu-804**—When Glu-804 was replaced with Asp, a smaller acidic amino acid, the resulting mutant retained responsiveness to high [Ca\textsuperscript{2+}]_i, equivalent to the wt CaR (Fig. 8a). Similarity, mutation of Glu to Gln produced a CaR with a signaling capacity comparable with the wt CaR (Fig. 8b). In contrast, cells expressing E804L and E804F CaR mutants did not respond to raising [Ca\textsuperscript{2+}]_i to 30 mM (Fig. 8a and b). Substitution to potentially basic amino acids (E804R and E804H) also produced CaR unable to increase InsPs even at the highest [Ca\textsuperscript{2+}]_i tested (Fig. 8, a and b). These results lent support to the potential importance of an acidic residue at position 804.

The expression of CaRs with different substitutions at positions 798, 802, and 804 was assessed by immunoblotting and immunocytochemistry. Immunoblotting showed that the expression patterns and levels of these mutant CaRs were comparable with those of the wt CaR (data not shown), except for the E804R CaR mutant, which showed an altered pattern of CaR protein expression. Immunoblotting revealed markedly reduced and, in three experiments, the lack of detectable expression of the expected ~160-kDa protein band in cells expressing this mutant. The expression of the ~140-kDa protein was relatively intact (Fig. 9). Similar findings were obtained from membranes prepared from four different transfections with two different mutant cDNA constructs. In these experiments, blotting with anti-CaR antiserum revealed bands of comparable intensity at ~140 and 160 kDa in cells expressing wt CaRs (data not shown).

To examine whether the signaling defects seen with CaR mutants at Leu-798, Phe-802, and Glu-804 were due to changes in cell surface expression of mutant receptors, we performed immunocytochemistry detecting signals with fluorescein and 3,3′-diaminobenzidine staining. In cells expressing wt CaRs (Fig. 10a), we consistently observed abundant receptor staining on the cell surface (arrowheads) and punctate staining of small vesicles (arrows) and aggregated staining apparently within peri-nuclear organelles (double arrows), possibly endoplasmic reticulum and Golgi. The same pattern was observed in cells expressing the CaRs mutated at positions 798 and 802 (Fig. 10, b and c, and data not shown). The localization of receptor-
specific staining, however, varied considerably in cells expressing CaRs mutated at position 804. The pattern of CaR immunostaining in cells expressing E804D and E804Q CaRs, which activated PLC comparable with the wt CaR, was equivalent to wt CaR-expressing cells (Figs. 10, d and e). In contrast, cell surface staining was substantially reduced in the cells expressing the E804A, E804F, E804H, E804L, and E804R CaRs, all of which appeared to be unable to activate PLC even at 30 mM Ca\(^{2+}\) (Fig. 10, f–j, respectively). In these cells, staining of perinuclear organelles was dramatically increased compared with membrane staining (Fig. 10, a versus f–j). These observations suggested that the apparent signaling defects of these CaR mutants were likely due to lack of surface expression.

**DISCUSSION**

The CaR belongs to the family 3 of GPCRs and, thus, shares modest sequence homology with the mGluRs, a large group of pheromone receptors, and the GABAB receptor. The second IC loops of these receptors are diverse, whereas the third IC loops of CaRs are up to 85% identical to their counterparts in mGluR1–8 and pheromone receptors (see Fig. 1b). These domains are critical in other receptors in mediating G-protein activation and signal transduction (10, 16, 21). To explore the functional roles of specific amino acids in these domains of the CaR, we mutagenized residues in the second and third IC loops of the bovine parathyroid CaR. Our results indicate that both loops contain sites important in PLC activation by this receptor. Specifically, hydrophobic, charged, and aromatic side chains of amino acids at key positions play critical roles in signal transduction and in mediating efficient receptor expression. Further work will be required to pinpoint contact points between the CaR and G-protein subunits or other effector molecules directly involved in mediating responses in target cells.

Our analysis of TA mutants of the CaR second IC loop revealed that a span of 8 residues in the amino terminus of this loop (700–707) was important in PLC activation. Mutagenesis to Ala of residues 700–703 and 704–707 in separate TA constructs produced CaR mutants with signaling-defective phenotypes. Mutating residues in the mid- and carboxyl-terminal portion of this loop, however, produced mild or no effects on signal transduction. In contrast to the CaR, the carboxyl-terminal end of the second IC loop is required for the PLC activation by the related mGluR1 (16). Point mutations of single residues in the 2 TA constructs (700–707) further identified two residues important for signaling: Leu-704 and Phe-707. Mutation of Leu-704 to Ala altered the ligand sensitivity of the CaR by shifting the ED\(_{50}\) for [Ca\(^{2+}\)] to the right. Mutating Phe-707 to Ala, however, had more dramatic effects. This residue turned out to be absolutely essential for coupling of the CaR to G-protein-dependent activation of PLC. There was es-

**FIG. 10.** Fluorescence immunocytochemistry of cells transiently expressing wt and mutant CaR cDNAs as indicated using an anti-CaR antisera and confocal microscopy as described under "Experimental Procedures." a, wt CaR; b, L798A CaR; c, F802A CaR; d, E804Q CaR; e, E804D CaR; f, E804A CaR; g, E804F CaR; h, S804H CaR; i, E804L CaR; j, E804R CaR; and k, untransfected control cells.

**FIG. 11.** Predicted helical conformation in the amino-terminal portion of the second IC loop in CaR. The secondary structure was predicted according to Chou-Fasman method using PeptideStructure and plotted using PlotStructure modules of Wisconsin Package V.10 software (Genetics Computer Group, Madison, Inc., WI).
sentially no tolerance for substituting this residue with other amino acids except for Tyr. These findings support the hypothesis that the phenyl side chain of Phe-707 may be part of a key interaction site of the CaR with signaling molecules and that Leu-704 may affect that or another interaction. Alternatively, both of these residues may be involved in maintaining a critical receptor conformation that allows for efficient signal transduction by the CaR.

In contrast to findings with L704A CaR noted above, mutation of Leu-705 to Ala, a Leu immediately adjacent to Leu-704, did not affect the ability of this CaR mutant to increase InsPs in response to high [Ca\(^{2+}\)\(_0\)]. This curious finding suggested that the primary and secondary structure of Leu at position 704 was critical for signaling by the CaR and that Leu-705 did not substitute for it. Modeling the secondary structure of the CaR second IC loop using the Chou-Fasman method (22) predicts that the amino terminus (i.e., residues 701 through 711) adopts an \(\alpha\)-helical conformation as illustrated in Fig. 11. According to this modeling, Leu-704 is brought closer to Phe-707 than is Leu-705 because of a helical turn. If Leu-704 and Phe-707 are physically closer, then the nature of the residue at 704 could have a greater impact on the role played by Phe-707, which our studies show to be absolutely critical in mediating CaR signaling via PLC. Whether this \(\alpha\)-helical model explains the results of our mutagenesis and whether this is the conformation important for G-protein activation by the CaR in intact cells remains unproven.

In examining the residues in the second IC loops of other members of family 3 receptors, we made 2 observations. The first was that there is striking sequence divergence among these loops with a few important exceptions (Fig. 1b). The critical Phe at position 707 in the parathyroid CaR is conserved in mGluR2, -3, -4, -6, and -8 and all known CaRs. These mGluRs preferentially couple to adenylate cyclase. A comparable Phe is not present in mGluR1 and -5, which activate PLC. This suggests that Phe-707 in the CaR and the analogous residue in mGluRs may not themselves participate in the direct activation of G-proteins, since G\(_s\) subunits are the ones that typically couple to PLC, and G\(_i\) and G\(_o\) subunits couple to adenylate cyclase. Instead, this Phe may be important in determining other critical aspects of receptor conformation necessary for signal transduction. We cannot rule out the possibility that this Phe may be important in the activation of G-protein subunits, which in some systems couple to effectors other than PLC, such as adenylate cyclase, in the case of mGluR2–4 and mGluR6–8, for example. The second observation we made was that, whereas Leu-704 and comparably positioned Leu are conserved among known CaRs, there are no analogous Leu residues in the mGluRs or GABA\(_\beta\) receptor. The analogous residues in the mGluRs or GABA\(_\beta\) receptor are Ala, Tyr, or His. Both Tyr and His differ significantly from Leu. Furthermore, modeling of the mGluRs and the GABA\(_\beta\) receptor suggest that they do not form \(\alpha\) helices in the amino-terminal regions of their second IC loops. These observations suggested that structural requirements for PLC activation in the second IC loop of the CaRs could potentially differ from those in other family 3 receptors.

Mutagenesis of residues in the third IC loop produced two types of signaling-defective mutants. The first group of mutants had mild signaling defects and included constructs in which Lys-794, Arg-796, Lys-797, Pro-799, Asn-801, Asn-803, Lys-806, or Phe-807 were converted to Ala. The capacity of these mutants to activate PLC was reduced by 35 to 65%, compared with wt responses. Although the defects of these mutant CaRs were most evident at [Ca\(^{2+}\)\(_0\)] \( \geq 10 \text{ mM}, \) their sensitivity to physiologic [Ca\(^{2+}\)\(_0\)] was also reduced, with a shift to the right in the ED\(_{50}\) for Ca\(^{2+}\) from \( \sim 5 \) to \( \sim 6 \) to 8 mM Ca\(^{2+}\). Four of these residues are basically charged amino acids (three Lys, one Arg). In other receptors, basic residues clearly participate in G-protein activation (5, 23–25). By immunoblotting and confocal microscopy, these mutants were expressed at levels comparable with wt CaR.

The second group of mutants included L798A, F802A, and E804A CaRs. These mutants were unable to activate PLC even at 30 mM Ca\(^{2+}\), indicating the potential importance of these three residues in coupling CaRs to downstream signaling molecules. L798A and F802A CaRs were expressed to similar extents as the wt CaR by Western blotting and by immunocytochemical analysis; these mutant receptors were strongly localized to the membrane. The mutant E804A CaR, however, appeared to be sequestered inside the cell with markedly reduced cell surface expression. Subsequent studies were directed at understanding the nature of the amino acid side-chain requirements at these sites for PLC activation and expression.

At position 798, only amino acids with nonpolar hydrocarbon side chains such as Ile, Val, and Phe could partially substitute for Leu and support signal transduction. Replacement of Leu-798 with the charged Glu or Lys or a less flexible Pro produced mutants unable to activate PLC. Clearly, the hydrophobicity of the residue at this position is critical for maintaining the receptor signaling function. The fact that hydrophobic amino acids (Val or Leu) are present at the corresponding positions in mGluR1 and mGluR4–8 and mammalian olfactory and GABAB receptors further underscores the importance of a hydrophobic side chain in signal transduction by family 3 GPCRs.

At position 802, the aromatic side chain of Phe also proved essential for CaR signaling via PLC. Only Tyr and, to a lesser extent, Trp at this position could reconstitute the ability of a CaR mutant to activate PLC fully. The critical nature of this Phe is underscored by the fact that this residue is conserved in the corresponding positions of the mGluR1–8 and pheromone receptors. Francesconi and Duvoisin (5) report that mutation of this residue in the mGluR1 to either Ser or Pro blocked the ability of this receptor to couple to PLC activation. These observations support the importance of this Phe in signal transduction by family 3 of GPCRs but do not indicate its exact function.

One possibility for how Phe is involved in signal transduction involves the \(\pi\) face of its phenyl group. Due to their overall hydrophobic nature and quadrupole charge distribution on their \(\pi\) faces, aromatic Phe, Tyr, and Trp can potentially stabilize positive charges of organic or inorganic cations in a non-aqueous environment (26, 27). This unique \(\pi\)-cation interaction provides an additional intermolecular force in mediating biological processes including enzyme activation, channel opening, and ligand-receptor interactions (26, 27). Whether the \(\pi\)-electron of Phe-802 plays a role in coupling of CaR to another amino acid in the receptor or in a G-protein subunit remains speculative. Another possibility is that this Phe maintains a secondary/tertiary structure in the third loop that somehow facilitates receptor activation of G-protein through other sites.

In contrast to the residues at positions 798 and 802, which maintain the receptor signaling function, a negatively charged amino acid is clearly preferred at position 804 to assure adequate membrane expression. Substitution of Asp for Glu at this position produced a mutant whose level of membrane expression and signaling capability were comparable with the wt CaR. Perhaps unexpectedly, a charged residue at position 804 was not required for the CaR to be expressed and activate PLC normally, since neutral Gln could substitute for Glu at this site. In the mGluR1–8, Glu residues are conserved in the corre-
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responding positions in their third IC loops. Mutation of this residue to Gln in the mGluR1 also produced a receptor fully capable of activating PLC and adenylate cyclase (5). Therefore, properties other than the negative charge of Glu may be important for CaR expression and function. In comparing Glu and Gln, it is noteworthy that their molecular weights are identical, and they both have carbonyl groups. This functional group can form a hydrogen bond (as a H\textsuperscript{+} acceptor) (28). It is doubtful that the size of the side chain of Glu-804 is critical because Asp, with a smaller acidic side chain, can substitute for Glu at this position. In addition, mutation of Glu-804 to Leu, a nonpolar amino acid with a molecular weight similar to Glu and Asp, resulted in a receptor mutant, which was not adequately expressed on the membrane. These observations lend support to the idea that Glu-804 may interact with a residue through the formation of a hydrogen bond and that such an interaction may be key to efficient insertion and recycling of CaRs in the membrane. Further studies will clearly be necessary to address the mechanisms by which mutants at position 804 of the CaR remain sequestered intracellularly in HEK293 cells.

The alterations in signaling we observed with mutants of the CaR second and third IC loops could have resulted from subtle reductions in CaR protein synthesis and cell surface expression not detectable by immunoblotting and immunocytochemistry. We recognize that these techniques allow for visualization of differentially glycosylated forms of the CaR, which may be functionally important, but they are semi-quantitative in estimating receptor number. Clearly, some of the milder signaling defects we observed could be due to subtle changes in receptor expression not detected by immunoblotting. In the cases of receptors with severe defects, such as the mutants at positions 707, 798, and 802, our immunoblotting and immunocytochemistry data suggested that signaling defects were unlikely, due to global disturbances in CaR protein expression. The inability of these receptors to interact productively with downstream signaling molecules in the PLC pathway is more likely to be the reason for our findings.

Point mutations of the CaR are linked to the diseases familial benign hypercalcemia, neonatal severe hyperparathyroidism, and isolated hypoparathyroidism. One naturally occurring point mutation has been identified in the second or third IC loops of the CaR. This mutant with Arg-795 converted to Trp (Arg-796 in bovine CaR) is markedly reduced in its ability to form receptor number. Clearly, some of the milder signaling reductions in CaR protein synthesis and cell surface expression resulting in a receptor mutant, which was not adequately expressed on the membrane.

mobilize intracellular Ca\textsuperscript{2+} (Arg-796 in bovine CaR) is markedly reduced in its ability to activate PLC, which likely explains the reduced intracellular Ca\textsuperscript{2+} mobilization noted by Bai et al. (18) and the importance of this site in vivo.

Overall, our studies demonstrate that PLC activation by the CaR requires key residues in at least two IC loops, like many other GPCRs. In addition to PLC activation, high Ca\textsuperscript{2+} suppresses cAMP production, promotes the opening of ion channels, and releases intracellular Ca\textsuperscript{2+} in parathyroid cells or HEK293 cells expressing CaRs (1, 3, 29, 30). Studies of other GPCRs suggest that a single receptor can couple to different signaling pathways and that specific residues in the IC loops and carboxyl-terminal tail can modify the selectivity of coupling to effector pathways (10, 17, 24). Whether the signaling determinants critical for PLC activation, which we have identified, are also involved in coupling the CaR to other signaling pathways remains to be explored. The development of receptor mutants selectively uncoupled from specific signaling pathways will enable the eventual delineation of how extracellular Ca\textsuperscript{2+} modifies diverse cell functions in vivo.

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Amino Acids in the Second and Third Intracellular Loops of the Parathyroid Ca\textsuperscript{2+} -sensing Receptor Mediate Efficient Coupling to Phospholipase C

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