The calcium-sensing receptor (CaR) belongs to family C of the G-protein-coupled receptor superfamily. To date 14 activating mutations in CaR showing superfamily to Ca\textsuperscript{2+} have been identified in humans with autosomal dominant hypocalcemia. Four of these activating mutations are found in the Ala\textsubscript{116}–Pro\textsubscript{136} region of CaR, indicating that this part of the receptor is particularly sensitive to mutation-induced activation. This region was subjected to random saturation mutagenesis, and 219 mutant receptor clones were isolated and screened pharmacologically in a high throughput screening assay. Selected mutants were characterized further in an inositol phosphate assay. The vast majority of the mutants tested displayed an increased affinity for Ca\textsuperscript{2+}. Furthermore, 21 of the mutants showed increased basal activity in the absence of agonist. This constitutive activity was not diminished when the mutations were transferred to a chimeric receptor Ca/1a consisting of the amino-terminal domain of the CaR and the 7 transmembrane and intracellular domains of the metabotropic glutamate receptor mGluR1a. CPCCOEt, a noncompetitive antagonist acting at the 7 transmembrane domain of mGluR1a, suppressed the elevated basal response of the constitutively activated Ca/1a mutants demonstrating inverse agonist activity of CPCCOEt. Taken together, our results demonstrate that the Ala\textsubscript{116}–Pro\textsubscript{136} region is of key importance for the maintenance of the inactive conformation of CaR.

The calcium-sensing receptor (CaR)\textsuperscript{1} belongs to family C of the 7 transmembrane (7TM) G-protein-coupled receptors (GPCRs) (1, 2). Besides CaR, the family comprises eight metabotropic glutamate receptors (mGluRs1–8) (3, 4), two \(\gamma\)-aminobutyric acid type B receptors (GABA\textsubscript{B}R1–2) (5), and a subfamily of putative pheromone receptors (6). The receptors in this family consist of a peptide chain longer than those of previously identified GPCRs and share no amino acid sequence similarity with any of these. Most notable is the unusually long amino-terminal domain (ATD) of \(-500–600\) amino acids, which has been shown to contain the site of agonist binding of CaR (7, 8), mGluR1 (9, 10), and GABA\textsubscript{B}R1a (11, 12).

The ATDs of the family C receptors have limited amino acid sequence similarity with procarboxylic periplasmic binding proteins (PBPs), a family of proteins involved in the transport of nutrients into bacteria (13). Based on the crystal structure of one of these PBPs, the leucine/iso-leucine/valine-binding protein, we have previously proposed a tertiary molecular model of the ATD of mGluR1 (9). Recently, Pin and co-workers (11) have presented a model of the ATD of GABA\textsubscript{B}R1a based on the crystal structure of the leucine-binding protein. The ATD of the family C receptor is thought to be constituted by two globular lobes separated by a hinge region creating a cleft. Two pseudo-conserved amino acids, corresponding to Ser\textsuperscript{147} and Thr\textsuperscript{1202} in the leucine/iso-leucine/valine-binding protein, have been identified as agonist binding residues in mGluR1 and GABA\textsubscript{B}R1a (9, 11). Furthermore, the two corresponding amino acids in CaR, Ser\textsuperscript{147} and Ser\textsuperscript{1770}, have been shown to be involved in receptor activation (7). Based on the known mechanism of the PBPs (13), it has been suggested that agonist binding to these amino acid residues causes the ATD of the family C receptor to contract from an "open" inactive conformation to a "closed" active conformation (9, 11). However, it is still unresolved how the activation signal subsequently is transferred from the closed ATD through the 7TM domain to the intracellular G-proteins.

CaR plays a central role in the extracellular calcium homeostasis. The receptor is expressed in parathyroid, kidney, intestine, central nervous system, and several other tissues (14). The primary function of CaR is to sense and mediate the effects of even minute changes in extracellular Ca\textsuperscript{2+} concentrations into parathyroid hormone secretion and renal excretion (14). In accordance with this, mutations in the CaR gene have been shown to cause abnormalities in blood Ca\textsuperscript{2+} levels. Three inherited human disorders have been linked to these somatic mutations: familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism, which are caused by inactivating mutations in CaR, and autosomal dominant hypocalcemia, which has been associated with activating mutations in CaR (14). The identified genetic mutations in CaR are depicted in Fig. 1. As can be seen, almost all of the mutations are located in the first half of the ATD and in the 7TM domain. We found

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\textsuperscript{2}This work was supported by grants from the Danish Medical Research Council, H. Lundbeck A/S, the Lundbeck Foundation, and the Novo Nordisk Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{1}The abbreviations used are: CaR, calcium-sensing receptor; 7TM, seven transmembrane domain; GPCR, G-protein-coupled receptor; mGluR, metabotropic glutamate receptor; GABA\textsubscript{B}R, \(\gamma\)-aminobutyric acid type B receptor; ATD, amino-terminal domain; PBP, periplasmic binding protein; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylic acid ethyl ester; CA, constitutively active; WT, wild type; IP, inositol phosphate; HBSS, Hanks’ balanced saline solution.
it of particular interest that 4 of the 14 identified activating mutations are located in the Ala116–Phe128 region of CaR. Ala116–Phe128 constitutes the first part of a region, Ala116–Pro136, which according to alignments with PBPs forms a loop in the ATD (7, 9).

Previously, we have successfully used random saturation mutagenesis on muscarinic acetylcholine receptors to investigate regions involved in receptor activation (15, 16) and G-protein coupling (17–19). In this study we have applied this technique to investigate whether the Ala116–Pro136 region of CaR is indeed as sensitive to mutational induced activation as suggested by the pattern of genetic mutations. Furthermore, we have studied the pharmacology of activated CaR mutants in greater detail using chimeric CaR/mGluR1a receptors and the noncompetitive mGluR1 antagonist, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt).

**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, UK). CPCCOEt was purchased from Tocris (Bristol, UK). rCaR-pRK5 (2) and pmGR1 (20) were generous gifts from professor Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD) and professor Shigetada Nakanishi (Kyoto University, Kyoto, Japan), respectively. The pSI and pEGFP-N2 vectors were obtained from Promega (Madison, WI) and CLONTECH (Palo Alto, CA), respectively. tsA cells were a generous gift from Dr. Penelope S. V. Jones (University of California, San Diego, CA).

**Subcloning and Site-directed Mutagenesis of CaR**—CaR was transferred from the pRK5 vector to the pSI vector as described previously (7). A silent SalI restriction site was created in the coding sequence for CaR spanning from nucleotide numbers 408–413 using the Quick-change mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Construction of the CaR D1035-EGFP plasmid was done by subcloning CaR into pEGFP-N2 using the restriction enzymes XhoI (a unique flanking restriction enzyme) and ApaI (covering nucleotides 3103–3108 in CaR). Subsequently, the mutagenic regions of three constitutively active (CA) mutants (mutants 17, 37, and 122) were subcloned into CaR D1035-EGFP using the restriction enzymes XhoI and EcoRI. The construction of the chimeric receptor Ca/1a, consisting of the ATD of CaR and the transmembrane and intracellular domains of mGluR1a, has been described previously (7). The mutagenic regions of the CA mutants of CaR-pSI were subcloned into Ca/1a-pSI using the restriction enzymes XhoI and EcoRI. Amplified receptor DNAs were sequenced on an ABI Prism 310 using Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Warrington, UK).

**FIG. 2.** Random saturation mutagenesis of nucleotides 346–407 in CaR-pSI. A, design of a 15% doped primer (P2). During the incorporation of each nucleotide, there was a 15% chance of misincorporation. B, library construction strategy. A silent SalI site was introduced in nucleotides 408–413 in CaR-pSI. Polymerase chain reaction was performed with the primers P1 and P2. The nonmutagenic primer (P1) comprised nucleotide residues 405–427 in pSI, including the HindIII site. The P2 primer comprised nucleotide residues 332–421 in CaR, nucleotides 322–345 and 408–421 being nonmutagenic and nucleotides 346–407 being 15% doped. The polymerase chain reaction product was restricted with HindIII and SalI, and the HindIII-SalI inserts were ligated into the HindIII-SalI fragment of the CaR-pSI, yielding a population of mutant CaR-pSI. Competent DH5α cells were transformed with this ligation mix, and plasmid DNA was isolated from 219 individual colonies.
Mutated nucleotides are indicated as capital letters. Nucleotide number 408 (the bold G in the end of the sequences) was not subject to mutation. The total number of mutations of each nucleotide in the 25 mutants is given in the last row.

| Receptor | Nucleotide 346–408 sequence |
|----------|-----------------------------|
| WT       | gcc cag aac aaa atc gat tct tgg aac ctg gag gac gtc tgc aac acc tct tact gag cac atc gccG |
| 1        | --- --- --- G-- --- --- --- A- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 2        | --- --- --- G-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 3        | --- --- --- G-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 4        | --- --- --- C-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 5        | --- --- --- C-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 6        | --- --- --- G-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 7        | --- --- --- T-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 8        | --- --- --- T-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 9        | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 10       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 11       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 12       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 13       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 14       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 15       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 16       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 17       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 18       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 19       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 20       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 21       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 22       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 23       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 24       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |
| 25       | --- --- --- A-- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- A--- --- --- --- G |

Mutation frequency

| WT | 143 433 434 323 414 616 626 262 245 313 653 345 334 313 814 455 633 425 255 233 464 43-- |

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**Functional Importance of the Ala<sup>116</sup>–Pro<sup>136</sup> Region in CaR**

**Table I**
The nucleotide 346–408 sequence segments of WT CaR and the 25 mutants first prepared

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**Library Construction**—Random saturation mutagenesis was performed on the 21-amino acid region Ala<sup>116</sup>–Pro<sup>136</sup> in CaR-pSI (nucleotides 346–407). The principles of random saturation mutagenesis are given in Fig. 2. A polymerase chain reaction was performed using the primers P1 and P2. P1 was a 22-nucleotide-long, nonmutagenic primer that included the HindIII site in the pSI vector. P2 was a 90-nucleotide-long primer. It was 15% “doped” in the 62-nucleotide region of interest, meaning that 85% of the correct nucleotide and 5% of each of the three other nucleotides were used for each individual nucleotide during the synthesis of the primer. Furthermore, P2 had a SauI site in the same position as the silent SauI site in CaR-pSI. The polymerase chain reaction product was digested with HindIII and SauI, and the cassettes were ligated into the HindIII/SauI fragment of CaR-pSI yielding a mutant CaR-pSI library. Competent DH<sub>5</sub>a Escherichia coli cells (Life Technologies, Inc., Paisley, UK) were transformed with the ligation mix, and mutant CaR-pSI clones were individually amplified. Plasmid DNA was isolated from 219 clones with the QiAprep spin plasmid miniprep kit (Qiagen, Hilden, Germany) and used for sequencing and functional assays.

**Cell Culture**—NIH 3T3 cells and tsA cells (a transformed HEK 293 cell line (21)) were maintained at 37 °C in humidified 5% CO<sub>2</sub> incubator in Dulbecco’s modified Eagle’s medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% calf serum.

**Receptor Selection and Amplification Technology Assays**—NIH 3T3 cells were plated into 96-well plates 1 day before transfection using 10<sup>4</sup> cells/well. The cells were transfected with 2.5 ng of wild type (WT) or mutant CaR-pSI and 25 ng of β-galactosidase-pSI/well using Superfect (Qiagen) as a DNA carrier. One day after transfection, cells were split into poly-D-lysine-coated 3.5-cm wells containing a glass slide (MatTek Corp., Ashland, MA) in inositol-free Dulbecco’s modified Eagle’s medium with reduced concentrations of CaCl<sub>2</sub> (0.9 mM) and MgCl<sub>2</sub> (0.6 mM), supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 10% dialyzed calf serum, and 1 µCi/ml myo-[2-<sup>3</sup>H]inositol (Amersharm Pharmacia Biotech, Buckinghamshire, UK). 16–24 h later, the cells were washed with Hanks’ balanced saline solution (HBSS) and incubated at 37 °C for 20 min in HBSS. The buffer was removed, and the cells were incubated for 40 min in HBSS supplemented with 10 mM LiCl and various concentrations of CaCl<sub>2</sub> or 100 µM CPTcCoET. The reactions were stopped by exchanging the buffer with 500 µl of ice-cold 20 mM formic acid, and separation of total [<sup>3</sup>H]inositol phosphates was carried out by ion exchange chromatography as described previously (23). All IP experiments were performed in duplicate, and the results are given as the means ± S.E. of at least three independent experiments.

**Measurement of the Content of Ca<sup>2+</sup>** and Mg<sup>2+</sup> in HBSS—The endogenous concentrations of Ca<sup>2+</sup> and Mg<sup>2+</sup> in HBSS were measured by flame atomic absorption spectroscopy, using an AAnalyst 100 (Perkin-Elmer, Warrington, UK).

**Single Cell Fluorescence Measurement of WT and Mutant CaR Expression**—3 × 10<sup>5</sup> tsA cells were split into a 6-cm tissue culture plate and transfected with 1.7 µg of plasmid the following day using Superfect as a DNA carrier according to the protocol by the manufacturer (Qiagen). The day after transfection, the cells were split into 12 wells in a poly-l-lysine-coated 24-well tissue culture plate in isoinitol-free Dulbecco’s modified Eagle’s medium with reduced concentrations of CaCl<sub>2</sub> (0.9 mM) and MgCl<sub>2</sub> (0.6 mM), supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 10% dialyzed calf serum, and 1 µCi/ml myo-[2-<sup>3</sup>H]inositol (Amersharm Pharmacia Biotech, Buckinghamshire, UK). The day after transfection, the cells were split into 12 wells in a poly-l-lysine-coated 24-well tissue culture plate in isoinitol-free Dulbecco’s modified Eagle’s medium with reduced concentrations of CaCl<sub>2</sub> (0.9 mM) and MgCl<sub>2</sub> (0.6 mM), supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% dialyzed calf serum. The following day, single cell fluorescence was viewed with an Axiosvert 100M (Zeiss, Jena, Germany) using the objective Plan-Apochromat 63 × 14 W Oil (DiC) and an excitation wavelength of 488 nm (emission maximum 507 nm).

**Data Analysis**—Data from the Ca<sup>2+</sup> concentration-response experiments were fitted to the simple mass action equation as follows.

\[
R = \frac{R_{\text{max}}}{1 + (EC_{50}/[A])^n} + R_{\text{baseline}}
\]

where [A] is the concentration of agonist, n is the Hill coefficient, and R is the response. Curves were generated by nonweighted least squares
can be described by the binomial function; \( b(25,0.15) \). H0: the mutations of each individual nucleotide in these 25 mutants agree with the theoretical mean of 3.75. The number of mismatches in these 25 mutants is 3.6 ± 6 S.D. of number of mutations of an individual nucleotide in these 25 mutants is 3.6 ± 1.4, which is in excellent agreement with the theoretical mean of 3.75. The number of mutations of each individual nucleotide in these 25 mutants can be described by the binomial function; \( b(25,0.15) \). H0: \( p = 0.15 \) was accepted, when tested both with \( n = 1 \) and \( n = 8 \). Hence H: \( p \neq 0.15 \) could not be proven for any of the 62 nucleotides. Consequently, it is reasonable to conclude that this library consists of mutant receptors being randomly mutated in the nucleotide 346–407 region.

Screening of the Mutant Library—Plasmid DNA was prepared from 219 individual clones, and the mutants were screened for pharmacological activity in R-SAT\textsuperscript{TM} (15–19). Both \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) were present in low concentrations (0.9 mM \( \text{MgCl}_2 \) and 0.8 mM \( \text{CaCl}_2 \)) at basal conditions, because of their importance for proper cell adhesion and function. These concentrations of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) did not activate WT CaR (Fig. 3). This is in agreement with previous results using, for example, IP measurements (7, 25). As exemplified by \( \text{Mg}^{2+} \) in Fig. 3, both agonists showed robust responses on CaR transfected cells in R-SAT\textsuperscript{TM}, when the concentration was increased. However, we decided to use \( \text{Mg}^{2+} \) for the library screening because \( \text{Ca}^{2+} \) showed a significant effect on mock-transfected NIH 3T3 cells at concentrations higher than 1.5 mM (data not shown). This was not the case with submaximal concentrations of \( \text{Mg}^{2+} \) (Fig. 3).

The screening data for a representative collection of mutants are given in Fig. 3. Based on the screening, the mutants could be divided into “WT-like” mutants, mutants with increased basal activity, and impaired/nonfunctional mutants. 78 mutants with either a significantly increased basal activity or a response comparable with that of WT CaR were selected for further pharmacological characterization.

Pharmacological Characterization of the CaR Mutants—The pharmacological characterization was performed using PI hydrolysis as a functional assay. In this assay exposure of mock-transfected tsA cells to \( \text{Ca}^{2+} \) at concentrations up to 10 mM did not give rise to any significant increase in IP accumulation (data not shown). In agreement with previous reports (7, 25), WT CaR displayed a 6–10-fold \( \text{Ca}^{2+} \)-induced IP stimulation (expressed as dpm/24-cluster well) in tsA cells transfected with WT and WT-like CaR mutants (A) or WT and CA CaR mutants (B). The cells were prelabeled overnight with 1 \( \mu \text{Ci/ml myo-2-3H} \)-inositol, washed with HBSS, and incubated with HBSS for 20 min. The buffer was removed, and the cells were incubated for 40 min in HBSS supplemented with 10 mM LiCl and various \( \text{CaCl}_2 \) concentrations. The buffer was aspirated, and the reactions were stopped by addition of ice-cold 20 mM formic acid. Total IP formation was determined by an ion exchange assay. Data are the means ± S.D. of duplicate experiments performed in parallel.

![Fig. 3. R-SAT\textsuperscript{TM} screening data for WT CaR and selected mutant CaRs.](image)

![Fig. 4. \( \text{Ca}^{2+} \) concentration-response curves for WT CaR and CaR mutants.](image)
responses as WT, whereas others were reduced. One explanation for reduced fold responses could be that basal responses are slightly increased in these mutants compared with WT (Fig. 4A). Whereas WT CaR reached its maximal stimulation at a \( \text{Ca}^{2+} \) concentration of 6.0 mM and remained at this level of response beyond this concentration, several of the "left-shifted" mutants displayed biphasic curves with a maximal response at 2.5–3.0 mM \( \text{Ca}^{2+} \) and a declining response at higher \( \text{Ca}^{2+} \) concentrations (Fig. 4A). This phenomenon has previously been reported by Brown and co-workers (26).

A considerable fraction of the mutants tested displayed a significantly higher level of basal IP accumulation than WT CaR, indicating that they might be constitutively activated (Fig. 4B and Table III). The mutants (termed CA CaR mutants) were still able to become activated in a concentration-dependent manner by \( \text{Ca}^{2+} \), although the Hill slopes of the mutants were significantly decreased compared with that of WT (Table III). These same mutants had previously exhibited an

### TABLE II

| Receptor | EC\textsubscript{50} (mM) | n\textsubscript{H} | Amino acid 116–136 sequence |
|----------|-----------------|----------|-----------------------------|
| WT       | 2.40 ± 0.08     | 4.6 ± 0.3| AQ K I D S L N L D F C N C S E H P |
| 54       | 0.78 ± 0.06     | 4.1 ± 0.3| R S K H V V E V |
| 154      | 0.88 ± 0.13     | 3.3 ± 0.4| R G V N |
| 84       | 0.92 ± 0.09     | 2.5 ± 0.3| G G G G |
| 71       | 0.97 ± 0.05     | 3.1 ± 0.4| V N G G |
| 33       | 0.97 ± 0.08     | 3.3 ± 0.5| H C F K |
| 155      | 0.97 ± 0.20     | 3.4 ± 0.7| A R R R |
| 176      | 1.01 ± 0.08     | 2.7 ± 0.3| H M K E S F D P |
| 125      | 1.02 ± 0.05     | 2.7 ± 0.5| T Q G Q L D Q Q |
| 62       | 1.03 ± 0.03     | 3.0 ± 0.5| N L K Y |
| 188      | 1.06 ± 0.08     | 3.3 ± 0.2| K T |
| 203      | 1.08 ± 0.01     | 3.6 ± 0.2| A M V G |
| 151      | 1.13 ± 0.04     | 3.0 ± 0.1| Q D S S F P R |
| 146      | 1.14 ± 0.10     | 2.9 ± 0.3| K V F |
| 93       | 1.16 ± 0.12     | 3.0 ± 0.3| K E F K D S |
| 206      | 1.17 ± 0.22     | 3.1 ± 0.3| G V M Q |
| 112      | 1.18 ± 0.05     | 3.2 ± 0.5| D Q |
| 128      | 1.20 ± 0.09     | 2.0 ± 0.4| E Q I V G K |
| 9        | 1.21 ± 0.08     | 3.3 ± 0.5| Q I W T K E |
| 57       | 1.22 ± 0.22     | 3.1 ± 0.3| H D R L F |
| 174      | 1.27 ± 0.07     | 3.4 ± 0.6| K N C R T E S |
| 90       | 1.54 ± 0.07     | 2.9 ± 0.4| K |
| 95       | 1.67 ± 0.15     | 3.2 ± 0.5| K V I S |
| 198      | 1.73 ± 0.05     | 3.9 ± 0.3| S F Q L |
| 14       | 1.40 ± 0.29     | 2.6 ± 0.4| N Q N M |
| 190      | 1.42 ± 0.12     | 3.0 ± 0.3| H L Y V |
| 170      | 1.42 ± 0.04     | 2.8 ± 0.5| G |
| 23       | 1.44 ± 0.08     | 3.7 ± 0.3| V D L Y Y W C A |
| 39       | 1.45 ± 0.07     | 4.4 ± 1.0| M P I T |
| 131      | 1.46 ± 0.13     | 4.6 ± 1.7| S T T Q H |
| 111      | 1.46 ± 0.00     | 4.2 ± 0.6| T F Y A S K |
| 123      | 1.46 ± 0.02     | 3.5 ± 0.3| K V E |
| 116      | 1.51 ± 0.17     | 3.1 ± 0.1| K E M A S N C |
| 143      | 1.54 ± 0.05     | 5.4 ± 0.6| S M E Y K |
| 61       | 1.55 ± 0.03     | 3.3 ± 0.5| D K L S V Y K L |
| 163      | 1.58 ± 0.05     | 4.0 ± 0.3| A F N S M |
| 6        | 1.60 ± 0.03     | 3.2 ± 0.7| V E |
| 183      | 1.61 ± 0.06     | 2.5 ± 0.2| S F K |
| 3        | 1.61 ± 0.09     | 3.2 ± 0.3| D |
| 124      | 1.62 ± 0.16     | 3.6 ± 0.2| K R R R |
| 72       | 1.64 ± 0.14     | 3.1 ± 0.3| K E F D I C Y |
| 204      | 1.67 ± 0.16     | 3.2 ± 0.4| F |
| 191      | 1.74 ± 0.03     | 2.9 ± 0.4| K M P Y S K N |
| 209      | 1.80 ± 0.04     | 3.6 ± 0.3| K S T E V H Y |
| 100      | 1.81 ± 0.17     | 4.3 ± 0.9| D L K C G |
| 7        | 1.83 ± 0.14     | 3.0 ± 0.4| E L R C |
| 5        | 1.84 ± 0.02     | 3.8 ± 0.2| D Q L S K |
| 82       | 1.87 ± 0.03     | 3.1 ± 0.5| L F T Y M |
| 180      | 1.95 ± 0.11     | 3.9 ± 0.4| Q Y S Y |
| 10       | 2.01 ± 0.20     | 3.8 ± 0.5| K Y V D A S T |
| 184      | 2.26 ± 0.17     | 3.9 ± 0.9| H T G M D C L |
| 172      | 2.28 ± 0.08     | 5.0 ± 0.7| T |
| 36       | 2.29 ± 0.16     | 4.4 ± 0.6| |
| 46       | 2.40 ± 0.11     | 4.5 ± 0.3| K L N I K A S |
| 135      | 2.51 ± 0.15     | 4.0 ± 0.3| K E M A S N C |
| 74       | 2.88 ± 0.11     | 4.5 ± 0.8| Y F G L D F |
| 197      | 3.18 ± 0.24     | 4.6 ± 0.4| Y T K E I Y L |
| 145      | 3.21 ± 0.11     | 4.0 ± 0.4| A E T T |

The CaR mutants with a basal response comparable with that of WT

The amino acid 116–136 sequence and pharmacological profile of those CaR mutants displaying a basal response comparable with that of WT in the inositol phosphate assay (less than 2.5-fold increase compared to WT). The assay was performed as described in Fig. 4, and the data are given as the means ± S.E. of at least three independent experiments. The mutants are listed after ascending EC\textsubscript{50} values. The sequence of mutant 36 was the same as WT. The average number of mutations in the mutants is 5.6 ± 1.6. A hyphen indicates that the amino acid is conserved in WT CaR.
increased basal response in R-SAT\textsuperscript{TM} (examples of these are given in Fig. 2).

**Measurement of Endogenous Ca\textsuperscript{2+} and Mg\textsuperscript{2+} Levels in HBSS**—To ensure that the CA mutants were not merely being activated by Ca\textsuperscript{2+} or Mg\textsuperscript{2+} in the HBSS, the content of these cations were measured in the buffer by flame atomic absorption spectroscopy. The endogenous concentrations of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} was determined to be below 150 ppb (3.8 \( \mu \)M) and 20 ppb (0.8 \( \mu \)M), respectively.

**Measurement of WT and Mutant CaR Expression**—The increased basal response of the CA CaR mutants could be due to an increased level of cell surface expression of the mutants compared with the WT receptor. To investigate this possibility, WT CaR and three of the CA mutants were subcloned into pEGFP-N2, which encodes a green fluorescent protein mutant (F64L,S65T). In a recent study of cell surface expression of carboxyl-terminal truncations of CaR fused in frame with EGFP, a CaR truncated at amino acid 886 displayed a pharmacology similar to that of WT CaR, when stably expressed in HEK 293 cells (27). Hence, it is reasonable to assume that the CaR\textsubscript{1035}-EGFP construct used in this study is representative for WT CaR.

In agreement with the study of Gama and Breitwieser (27), the fluorescence pattern of tsA cells transfected with EGFP-N2 and that of cells transfected with the CaR\textsubscript{1035}-EGFP construct turned out to be quite different (Fig. 2, A and B). Furthermore, the cell surface expression of the three CA mutants tested, CA17-, CA37-, and CA122-CaR\textsubscript{1035}-EGFP, were not that different from that of the CaR\textsubscript{1035}-EGFP (Fig. 5). In tsA cells transfected with pSI, no fluorescence was detected (data not shown).

Recent studies have revealed that several of the cysteines in the ATD of CaR are crucial for CaR expression, dimerization, and functionality (28, 29). The two cysteines in the Ala\textsubscript{116–Pro\textsuperscript{136}} region, Cys\textsuperscript{129} and Cys\textsuperscript{131} are among the few cysteines that are of little or no importance for receptor expression levels (28). In CA17-CaR\textsubscript{1035}-EGFP both of these cysteines have been mutated and as just mentioned the expression levels are comparable with WT (Fig. 5, B and C). However, when it comes to implications for receptor functionality, mutations of Cys\textsuperscript{129} and Cys\textsuperscript{131} clearly do not impair receptor function in any way (Tables II and III), which is in excellent agreement with the previous studies (28, 29).

**Pharmacological Characterization of Ca/1a and the CA Ca/1a Mutants**—The majority of the mutants displaying a significantly elevated basal response in the R-SAT\textsuperscript{TM} screen and in the IP assay were subcloned from CaR-pSI into Ca/1a-pSI. Ca/1a and Ca/1b are chimeric receptors consisting of the ATD of CaR and the 7TM region and carboxyl terminus of mGluR1a or mGluR1b, respectively. Both chimers have been shown to display pharmacological profiles very similar to that of the WT CaR (7, 8, 30). The reason for the transfer was that no antagonist for CaR has yet been published. By transferring the CA mutations to Ca/1a, we were able to use the noncompetitive mGluR1 antagonist CPCCOEt (31), which has been shown to bind to the extracellular side of TM7 of mGluR1 and
accumulation in tsA cells transfected with CaR1a and mutant CaR1a mutants. A. IP accumulation in tsA cells transfected with CaR1a and mutant CaR1a’s tested in the absence of Ca2+ (basal) and in the presence of 6.0 mM Ca2+. B, CPCCOEt inhibition of basal IP accumulation in tsA cells transfected with CaR1a and CA CaR1a mutants. The cells were prelabeled overnight with 1 μCi/ml myo-[2-3H]inositol, washed with HBSS, and incubated for 20 min in HBSS. The buffer was removed, and the cells were incubated for 40 min in HBSS supplemented with 10 mM LiCl and with or without the indicated concentrations of CaCl2 (A) or CPCCOEt (B). Finally, the buffer was aspirated, and the reactions were stopped by addition of ice-cold 20 mM formic acid. Total IP formation was determined by an ion exchange assay. IP accumulation is expressed as dpm/24-cluster well. Data are the means ± S.D. of duplicate experiments performed in parallel.

FIG. 6. Ca2+ induced IP stimulation and CPCCOEt inhibition of constitutive activity of CaR1a and CA CaR1a mutants. A, IP accumulation in tsA cells transfected with CaR1a and mutant CaR1a’s tested in the absence of Ca2+ (basal) and in the presence of 6.0 mM Ca2+. B, CPCCOEt inhibition of basal IP accumulation in tsA cells transfected with CaR1a and CA CaR1a mutants. The cells were prelabeled overnight with 1 μCi/ml myo-[2-3H]inositol, washed with HBSS, and incubated for 20 min in HBSS. The buffer was removed, and the cells were incubated for 40 min in HBSS supplemented with 10 mM LiCl and with or without the indicated concentrations of CaCl2 (A) or CPCCOEt (B). Finally, the buffer was aspirated, and the reactions were stopped by addition of ice-cold 20 mM formic acid. Total IP formation was determined by an ion exchange assay. IP accumulation is expressed as dpm/24-cluster well. Data are the means ± S.D. of duplicate experiments performed in parallel.

to antagonize Ca2+ mediated agonist responses by CaR1b (30, 32). CPCCOEt has also been shown to have no effect on Ca2+ induced activation of WT CaR in concentrations up to 100 μM (30).

The basal level of IP accumulation was not significantly reduced, when CaR1a transfected tsA cells were exposed to 100 μM CPCCOEt in the absence of Ca2+, indicating that the chimeric receptor is not constitutively active (Fig. 6B). In accordance with previous findings (7), CaR1a exhibited a 5–7-fold increase in IP accumulation, when exposed to 6.0 mM Ca2+ (maximal stimulation; Fig. 6A). In contrast, the mutants transferred from CaR1a maintained their high basal response as CaR1a mutants (termed CA CaR1a mutants), when compared with CaR1a (Fig. 6). They could all become further activated upon exposure to Ca2+ (Fig. 6A), and the elevated basal response of almost all the mutants could be inhibited with 100 μM CPC- COEt (Fig. 6B). A few of the mutants did not respond to 100 μM CPCCOEt (Fig. 6B). Because of the reported toxicity of CPC- COEt concentrations above 100 μM (30), it was not possible to study these mutants in greater detail using this ligand.

DISCUSSION

CaR is among the relatively few GPCRs for which genetic mutations have been linked to human disorders. Almost all of the identified somatic mutations in CaR are located either in the first half of the ATD or in the 7TM region of the receptor (Fig. 1). A number of at least 14 activating mutations in CaR have been found in individuals with autosomal dominant hypocalcemia (14, 33–36). Four of these are located in a region of only 13 amino acids in the ATD, Ala116–Phe128 (14). This pattern of genetic mutations indicates that the region is particularly sensitive to mutationally induced activation and thus could be involved in the activation mechanism of CaR. As pointed out previously, Ala116–Phe128 constitutes the first half of a region, Ala116–Pro136, which according to alignments with PDBs forms a loop in the ATD (7, 9). In the present study we subjected the entire Ala116–Pro136 region to random saturation mutagenesis.

We report that the Ala116–Pro136 region is in fact of key importance to the equilibrium between the inactive and the active state of the receptor. The left-shifted concentration-response curves observed for the majority of the mutants in this study are similar to the pharmacological profiles for several of the CaR mutations found in individuals with autosomal dominant hypocalcemia, including those with mutations in the Ala116–Phe128 region (26, 37–39). In addition to these left-shifted mutants, we have identified a considerable number of constitutively active mutants (10% of the entire mutant library). The observation that the CA mutants displayed EC50 values in the millimolar range well above the low micromolar concentrations of Ca2+ and Mg2+ measured in the HBSS buffer shows that the elevated basal level is agonist-independent. Furthermore, the cell surface expression levels of a representative selection of these mutants are not significantly different compared with that of the WT receptor. Together, these observations clearly indicate that the mutants are truly constitutively active. Comparison of Tables II and III indicates that changes in Ca2+ potency and basal activity are independent of each other. Previously, we have found these two pharmacological parameters to be highly correlated in the m5 muscarinic acetylcholine receptor (40), whereas Kjelsberg et al. (41), in analogy with the present results, also reported the two pharmacological parameters to be independent of each other on a series of mutants in the α1A-adrenergic receptor.

We and others have previously described constitutively activating mutations in the 7TM region of family A GPCRs such as adrenergic (41, 42) and muscarinic acetylcholine receptors (15, 16, 40). In the same studies it was found that some antagonists previously thought merely to act as agonist blockers were able to decrease the elevated basal activity, and thus these ligands were reclassified as inverse agonists according to generally accepted definitions (43). Constitutive activity and inverse agonism are best explained by a two-state model in which receptors equilibrate between an inactive and an active conformation. According to this model agonists and inverse agonists stabilize the active and an inactive receptor conformation, respectively, and thus shift the equilibrium accordingly. Mutations conferring constitutive activity are thought to change the equilibrium constant to favor the active conformation thus leading to agonist-independent basal activity (40, 42).

Of the 14 activating CaR mutations reported today, only A843E located in the 7TM region (Fig. 1) has been reported to
cause agonist-independent increase of the basal level (35). To our knowledge, the present study is the first report of mutation-induced constitutive activity in the ATD of a family C receptor. Interestingly, the mutations are located in the ATD rather than in the TM7 region as previously reported. Based on the homology of the ATDs with the PBPs and the known mechanism of the latter, we have previously suggested that the initial event in family C receptor activation is transformation of the open ATD into a closed agonist-bound ATD (9). This is in agreement with the two-state model in which the open and closed form of the ATD would represent the inactive and active receptor conformation, respectively.

As pointed out previously (4, 7, 30), the mechanism by which the signal is transferred from the ATD through the TM7 to the intracellular G-proteins is unknown. However, several models of “signal transference” have been suggested (4, 9, 35). Agonist binding to and activation of the ATD can be speculated to initiate 1) a transference of the agonist from the ATD to a second agonist site in the TM7 followed by a “classical” TM7 conformational change, 2) a specific interaction between regions in the ATD and the TM7 of the receptor inducing a conformational change in the latter, or 3) a conformational change in the entire receptor protein, enabling the G-protein coupling. Because the chimeric receptors Ca1a and Ca1b have been shown to have almost identical pharmacological profiles as WT CaR (7, 8, 30), the existence of a second Ca2+-binding site in the TM7 of CaR is very unlikely. Following the same reasoning and considering the low amino acid sequence similarity between the CaR and mGluR1, it is also hard to imagine regions in the ATD of CaR and the TM7 of mGluR1 interacting in a specific manner, although this possibility cannot be entirely excluded. In any case, in consideration of the lack of sequence similarity between the Ala116–Pro136 region in CaR and the corresponding region in the mGluR1, Arg124–Pro155 (7), it seems almost impossible that residues in the Ala116–Pro136 region could substitute amino acids in the corresponding mGluR1 region and function as an “intramolecular agonist” involved in a specific interaction with the TM7 of mGluR1. The fact that constitutive activity in the CA CaR mutants can be transferred to the Ca1a receptor indicates that the activating effect created by the mutations can be ascribed solely to structural changes arising within the ATD of CaR. In this context it is interesting to note the absence of specific mutations or mutational patterns causing constitutive activity by comparing Tables II and III. Based on studies of constitutively activating mutations in the α1B-adrenergic (41) and m5 muscarinic receptors (40), it has been suggested that GPCRs are constrained in the inactive conformation and that mutational induction of constitutive activity is caused by disruption of the inactive receptor conformation. The fact that the vast majority of mutants in the study showed left-shifted concentration-response curves, a large fraction of them being constitutively activated, is consistent with this hypothesis. Thus, the Ala116–Pro136 region appears to be important for constraining the ATD of CaR in the inactive conformation. Mutations within the region relieve these constraints, favoring the active conformation through the same activation mechanism of unknown nature as that induced by agonists on the WT CaR.

Based on the results from our study of Ser145 (located on the extracellular side of TM6) in the m5 muscarinic acetylcholine receptor, we have previously suggested that this region could be a potential site of drug action because ligands acting at this site could mimic the mutations and thus increase the affinity of the endogenous agonist (40). In analogy with this, it seems likely that ligands acting at the Ala116–Pro136 region could act as allosteric activators/agonists. Although the site of action remains to be determined, compounds such as NPS R-467 and NPS R-568 have been shown to be allosteric activators of CaR (44), and compounds with this pharmacological profile have shown potential as agents for the treatment of primary hyperparathyroidism (45, 46). In light of the results presented in this study, it should be interesting to study the mechanism of action of these compounds in greater detail.

In agreement with previous studies using mGluR1 and mGluR5 chimers (32), we have recently shown that the non-competitive mGluR1 antagonist CPCCOEt interacts with the TM7 region of the receptor (30). In the present study we extended this finding by showing that CPCCOEt acts as an inverse agonist. This observation is in agreement with recent findings of Pin and co-workers,2 where it was shown that CPCCOEt acts as a partial inverse agonist on the increased basal activity generated by co-transfection of cells with mGluR1 and the G-protein Gq. The basal response of the few CA Ca1a mutants that were not affected by 100 μM CPCCOEt could perhaps have been suppressed with higher concentrations of this ligand. However, because of the reported toxicity of CPCCOEt at concentrations higher that 100 μM (30), we were unable to study these “nonresponsive” CA mutants in greater detail using of this ligand. On the other hand, our results clearly demonstrate that chimeric CA receptors can be used as a tool to determine whether compounds acting at the TM7 region are inverse agonists. Given that a growing number of noncompetitive family C receptor antagonists are being identified (32, 47–50), of which several have been shown to act at the TM7 region, chimeric CA receptors such as those presented in this study, may prove to be valuable tools for studies of these compounds.

Acknowledgments—We thank Drs. Solomon Snyder, Shigetada Nakanishi, and Penelope S. V. Jones for the kind gifts of plasmas and cell lines. We also acknowledge Drs. Ole Jens and Birger Brolin for technical assistance with the flame atomic absorption spectroscopy and the single cell fluorescence measurements, respectively.

Note Added in Proof—In agreement with our results, a very recent follow up study by the group of Dr. Spiegel (Ray, K., Hauschild, B. C., Steinbach, P. J., Goldsmith, P. K., Hauoche, O., and Spiegel, A. M. (1999) J. Biol. Chem. 274, 27642–27650) has shown that Cys129 → Ser, Cys131 → Ser, and Cys133 → Ser mutants display 2–4-fold lower EC50 values for Ca2+ than WT CaR.

REFERENCES

1. Brown, R. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifir, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993) Nature 366, 575–580
2. Ruat, M., Molliver, M. E., Snowman, A. M., and Snyder, S. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3161–3165
3. Nakanishi, S. (1994) Neuron 12, 1031–1037
4. Pin, J. P., De Colle, C., Bessis, A. S., and Acher, F. (1999) Eur. J. Pharmacol. 375, 277–294
5. Mohler, H., and Frischmeyer, J. M. (1999) Trends Pharmacol. Sci. 20, 87–89
6. Tirindelli, R., Mucignat-Caretta, C., and Ryba, N. J. (1999) Trends Neurosci. 21, 482–486
7. Brauner-Osborne, H., Jensen, A. A., Sheppard, P. O., O’Hara, P., and Krosggaard-Larsen, P. (1999) J. Biol. Chem. 274, 18382–18386
8. Hammerland, L. G., Krupcho, K. J., Garrett, J. E., Alasti, N., Hung, B. C., Simin, R. T., Levinthal, C., Nemeth, E. F., and Fuller, F. H. (1999) Mol. Pharmacol. 56, 642–646
9. O’Hara, P. J., Sheppard, P. O., Thagersen, H., Venezia, D., Haldeman, B. A., McGraw, V., Houamed, K. M., Thomsen, C., Gilbert, T. L., and Mulvihill, E. R. (1995) Neuron 11, 41–52
10. Okamoto, T., Sekiyama, N., Otsu, M., Shimada, Y., Sato, A., Nakanishi, S., and Jingami, H. (1998) J. Biol. Chem. 273, 13089–13096
11. Galvez, T., Parmentier, M. L., Joly, C., Malitshchek, B., Kaufmann, K., Kuhn, R., Bittiger, H., Froestl, W., Bettler, B., and Pin, J. P. (1999) J. Biol. Chem. 274, 13362–13369
12. Malitshchek, B., Schweizer, C., Keir, M., Heid, J., Froestl, W., Mosbacher, J., Kuhn, R., Henley, J., Lyon, J., Pin, J. P., Kaufmann, K., and Bettler, B. (1999) Mol. Pharmacol. 56, 448–454
13. Quiocho, F. A., and Ledvina, P. S. (1996) Mol. Microbiol. 20, 17–25
14. Brown, E. M. (1999) Am. J. Med. 106, 238–253
15. Spalding, T. A., Burstein, E. S., Brauner-Osborne, H., Hill-Eubanks, D., and

2 J.-P. Pin and L. Prézeau, personal communication.
21. Chahine, M., Bennett, P. B., George, A. L., Jr., and Horn, R. (1994) Pflugers Arch. 427, 136–142
22. Lim, K., and Chae, C.-B. (1989)
23. Nanevics, T., Wang, L., Chen, M., Ishii, M., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 3058–3065
24. Brauner-Osborne, H., and Brann, M. R. (1996)
25. Ruat, M., Snowman, A. M., Hester, L. D., and Snyder, S. H. (1996)
26. Pearce, S. H., Williamson, C., Kifor, O., Bai, M., Coulthard, M. G., Davies, M., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) J. Biol. Chem. 271, 19537–19545
27. Pollak, M. R., Brown, E. M., Estep, H. L., McLaIne, P. N., Kifor, O., Park, J., Hebert, S. C., Seidman, C. E., and Seidman, J. G. (1994) Nat. Genet. 8, 303–307
28. Spalding, T. A., Burstein, E. S., Wells, J. W., and Brann, M. R. (1997) Biochemistry 36, 10109–10116
29. Fan, G., Ray, K., Zhao, X., Goldsmith, P. K., and Spiegel, A. M. (1998) FEBS Lett. 436, 353–356
30. Pace, A. J., Gama, L., and Breitwieser, G. E. (1999) J. Biol. Chem. 274, 11629–11634
31. Okazaki, R., Chikatsu, N., Nakatsu, M., Takeuchi, Y., Ajima, M., Miki, J., Fujita, T., Arai, M., Totsuka, Y., Tanaka, K., and Fukushima, S. (1999) J. Clin. Endocrinol. Metab. 84, 363–366
32. De Luca, F., Ray, K., Mancrella, E. E., Fan, G. F., Winer, K. K., Gore, P., Spiegel, A. M., and Barno, J. (1997) J. Clin. Endocrinol. Metab. 82, 2710–2715
33. Zhao, X. M., Hauuche, O., Goldsmith, P. K., Collins, R., and Spiegel, A. M. (1999) FEBS Lett. 448, 180–184
34. Watanabe, T., Bae, M., Lane, C. R., Matsumoto, S., Minamitani, K., Minagawa, M., Niimi, H., Brown, E. M., and Yasuda, T. (1988) J. Clin. Endocrinol. Metab. 63, 2497–2502
35. Baron, J., Winer, K. K., Yanovsky, J. A., Cunningham, A. W., Laue, L., Zimmerman, D., and Cutler, G. B., Jr. (1996) Hum. Mol. Genet. 5, 601–606
36. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H. S., Pollak, M. R., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) J. Biol. Chem. 271, 19537–19545
37. Varney, M. A., Conford, N. D., Jachec, C., Rao, S. P., Sacaan, A., Lin, F. F., Bleicher, L., Santori, E. M., Flor, P. J., Allgeier, H., Gasparini, P., Kuhn, R., Hess, S. D., Veli elebi, G., and Johnson, E. C. (1999) J. Pharmacol. Exp. Ther. 290, 170–181
38. Gasparini, P., Lingenho¨hl, K., Stoehr, N., Flor, P. J., Heinrich, M., Vranesc, I., Biollaz, M., Allgeier, H., Heckendorn, R., Urwyler, S., Varney, M. A., Johnson, E. C., Hess, S. D., Rao, S. P., Sacaan, A. I., Santori, E. M., Velipelebi, G., and Kuhn, R. (1999) Neuropharmacology 38, 1483–1503
39. Ward, R. K., Stucky, B. G., Gutteridge, D. H., Laing, N. G., Pullan, P. T., and Ratajczak, T. (1999) Hum. Mutat. 10, 233–235
40. Kobayashi, M., Tanaka, H., Totsuka, Y., Takeuchi, Y., Takeda, S., Yamura, Y., Matsumoto, T., and Fujita, T. (1999) Clin. Endocrinol. Metab. 82, 2716–2719
41. Chikatsu, N., Fukushima, S., Suzawa, M., Tanaka, Y., Takeuchi, Y., Takeda, S., Yamura, Y., Matsumoto, T., and Fujita, T. (1999) Clin. Endocrinol. Metab. 82, 537–543