Protein Kinase C Phosphorylation of Threonine at Position 888 in Ca\(^{2+}\)\(_o\)-Sensing Receptor (CaR) Inhibits Coupling to Ca\(^{2+}\) Store Release*

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Mei Bai‡§, Sunita Trivedi‡, Charles R. Lane‡, Yinhai Yang‡, Steven J. Quinn‡, and Edward M. Brown‡

From the ‡Endocrine-Hypertension Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School and the ¶Department of Cardiology, Research Laboratory, Children's Hospital, Boston, Massachusetts 02115

Previous studies in parathyroid cells, which express the G protein-coupled, extracellular calcium-sensing receptor (CaR), showed that activation of protein kinase C (PKC) blunts high extracellular calcium (Ca\(^{2+}\)_o)-evoked stimulation of phospholipase C and the associated increases in cytosolic calcium (Ca\(^{2+}\)_i), suggesting that PKC may directly modulate the coupling of the CaR to intracellular signaling systems. In this study, we examined the role of PKC in regulating the coupling of the CaR to Ca\(^{2+}\)_i, dynamics in parathyroid cells. We demonstrate that several PKC activators exert inhibitory effects on CaR-mediated increases in Ca\(^{2+}\)_i due to release of Ca\(^{2+}\)_o from intracellular stores. Consistent with the effect being mediated by activation of PKC, the inhibitory effect of PKC activators on Ca\(^{2+}\)_i release can be blocked by a PKC inhibitor. The use of site-directed mutagenesis reveals that threonine at amino acid position 888 is the major PKC site that mediates the inhibitory effect of PKC activators on Ca\(^{2+}\)_o mobilization. The effect of PKC activation can be maximally blocked by mutating three PKC sites (Thr\(^{888}\), Ser\(^{895}\), and Ser\(^{915}\)) or all five PKC sites. In vitro phosphorylation shows that Thr\(^{888}\) is readily phosphorylated by PKC. Our results suggest that phosphorylation of the CaR is the molecular basis for the previously described effect of PKC activation on Ca\(^{2+}\)_o-evoked changes in Ca\(^{2+}\)_i dynamics in parathyroid cells.

The extracellular calcium concentration (Ca\(^{2+}\)_o) is tightly regulated by the interactions of several hormones (e.g. parathyroid hormone (PTH), vitamin D, and calcitonin) and organ systems (i.e. parathyroid gland, kidney, bone, and intestine) (1). Parathyroid cells respond to changes in Ca\(^{2+}\)_o, with oppositely directed alterations in PTH secretion through a cell surface, G protein-coupled receptor, the Ca\(^{2+}\)_o-sensing receptor (CaR).

The CaR was first isolated from bovine parathyroid cells using expression cloning in Xenopus laevis oocytes and shows pharmacological properties nearly identical to those of the native receptor in its responses to agonists such as extracellular divalent cations (e.g. Ca\(^{2+}\)_o and Mg\(^{2+}\)_o), trivalent cations (e.g. Gd\(^{3+}\)_o) and polyamines (e.g. neomycin) (2). In response to increases in Ca\(^{2+}\)_o, the CaR stimulates accumulation of inositol phosphates and produces transient followed by sustained increases in Ca\(^{2+}\)_i. Subsequently, cDNAs encoding the human homologue of the same receptor have been cloned from parathyroid (3) and kidney (4) using a homology-based strategy. The physiological relevance of the CaR for mineral ion metabolism has been documented by the identification of CaR mutations in patients with inherited disorders of calcium homeostasis (36, 37).

High Ca\(^{2+}\)_o-evoked suppression of PTH secretion and the concurrent increases in Ca\(^{2+}\)_i in parathyroid cells can be negatively regulated by activation of protein kinase C (PKC) (5–13) Such negative regulation by PKC has been suggested to be involved in the reduced responsiveness of adenomatous or hyperplastic parathyroid glands to Ca\(^{2+}\)_o, as a result of an increase in membrane-associated PKC (14, 15), although there is also reduced expression of the CaR in these glands (16, 17). Likewise, PKC may contribute to age-related changes in the regulation of PTH secretion by Ca\(^{2+}\)_o in rats (18). Therefore, stimulus-secretion coupling in parathyroid cells can be modulated by PKC, perhaps at an early step in the process of Ca\(^{2+}\)_o sensing.

The human homologue of the CaR is predicted to have five PKC sites in its intracellular domains. We hypothesized that PKC modulates the sensitivity of parathyroid cells to changes in Ca\(^{2+}\)_o by covalently modifying these sites. To test this hypothesis, we have transiently transfected a human parathyroid CaR cDNA (18) in HEK293 cells and mutated each of the five predicted PKC phosphorylation sites (Thr\(^{888}\), Ser\(^{895}\), and Ser\(^{915}\)) or all five PKC sites. In vitro phosphorylation shows that Thr\(^{888}\) is readily phosphorylated by PKC. Our results suggest that phosphorylation of the CaR is the molecular basis for the previously described effect of PKC activation on Ca\(^{2+}\)_o-evoked changes in Ca\(^{2+}\)_i dynamics in parathyroid cells.

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‡ To whom correspondence should be addressed: Endocrine-Hypertension Division, Brigham and Women's Hospital, 221 Longwood Ave., Boston, MA 02115. Tel.: 617-732-4093; Fax: 617-732-5764.

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The abbreviations used are: PTH, parathyroid hormone; CaR, extracellular calcium-sensing receptor; PKC, protein kinase C; PMA, phorbol myristate acetate; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholino-2-propanesulfonic acid; bp, base pairs; Ca\(^{2+}\)_o, extracellular calcium.

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or perhaps generation of some other intracellular mediator(s) along the inositol trisphosphate/phospholipase C pathway.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the approach described by Kunkel (19) to produce mutated receptors in which one or more serine or throne residue present in the five predicted PKC phosphorylation sites within intracellular domains of the human CaR were mutated to alanine or valine, respectively. The δt-1 ung-1 strain of Escherichia coli (DH5α) was transformed separately with mutagenesis cassette 5 or 6, as described previously (20). For two receptors with two mutations each (T888V/Ha915V) and one with three mutations (T888V/Ha915V/S916V), the two fragments (168 and 795 bp) obtained from the above digestions were ligated to the large fragment resulting from digestion of the wild type CaR in pcDNA3 with XhoI. The resultant clone was confirmed by sequencing to carry these two mutations.

**Construction of a Mutant CaR with Mutations of Two PKC Sites** (S895A/S915A)—Cassette 6 was doubly digested with XhoI and XbaI, and the same cassette carrying three mutated PKC sites was doubly digested with HpaI and XhoI and cloned into the reconstructed receptor in pcDNA3 (Invitrogen), as described previously (20). Likewise, mutated versions of cassette 6 containing the desired mutations were doubly digested with XhoI and XbaI and cloned into the reconstructed receptor in pcDNA3.

**Construction of a Mutant CaR with Mutations of Two PKC Sites** (T646V/S794A)—The mutant receptor carrying T646V was doubly digested with HpaI and XhoI, and the mutant receptor carrying S794A was doubly digested with XhoI and XbaI. Two small fragments (404 and 568 bp) obtained from the above digestions were ligated to the large fragment resulting from digestion of the parent reconstructed CaR clone with XhoI. The resultant clone was confirmed by sequencing to carry these two mutations.

**Construction of a Mutant CaR with Mutations of Two PKC Sites** (T646V/S794A) The mutant receptor carrying T646V was doubly digested with HpaI and XhoI, and the mutant receptor carrying S794A was doubly digested with XhoI and XbaI. Two small fragments (404 and 975 bp) obtained from the above digestions were ligated to the large fragment resulting from digestion of the wild type CaR in pcDNA3 with HpaI and XhoI. The resultant clone was confirmed by sequencing to carry these two mutations.

**Construction of a Mutant CaR with Four Mutated PKC Sites** (T646V/S794A/ T888V/Ha915V)—The mutagenesis cassette carrying four mutated PKC sites and two mutated sites (S895A/S915A) was doubly digested with KpnI and XhoI to obtain the full-length CAR inserts, which were further digested with SpI. One fragment (2434 bp) containing T646V/S794A and another fragment (803 bp) containing S895A/S915A, obtained from the above digestions, were ligated to pcDNA3 generated by KpnI and XbaI. The resultant clone was confirmed by sequencing to carry all five mutations.

**Construction of a Mutant CaR with Four Mutated PKC Sites** with Thr646 unchanged (T646V/S794A/ S895A/S915A)—The receptors carrying five mutated PKC sites and two mutated sites were doubly digested with XbaI and XhoI to obtain the full-length CAR inserts, which were further digested with SpI. One fragment (2434 bp) containing T646V/S794A and another fragment (803 bp) containing S895A/S915A, obtained from the above digestions, were ligated to pcDNA3 generated by KpnI and XbaI. The resultant clone was confirmed by sequencing to carry four mutations.

**Construction of Flag-tagged CaR**—The Flag, an epitope tag, was introduced into the third cassette of the wild type CaR as described previously (21). The third cassette containing Flag was ligated with AflIII and NheI and ligated to the large fragments resulting from digestion of the CaRs containing PKC site mutations.

**Transient Expression of CaRs in HEK293 Cells**—CaR cDNAs were prepared using the Midi Plasmid Kit (Qiagen). LipofectAMINE (Life Technologies, Invitrogen) was employed as a DNA transfection. The transient expression of HEK293 cells used for transient transfection were provided by NPS Pharmaceuticals, Inc. (Salt Lake City, UT) and were cultured in DMEM (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone). The DNA-liposome complex was prepared by mixing DNA and LipofectAMINE in Opti-MEM I reduced serum medium (Life Technologies, Inc.) and incubating the mixture at room temperature for 30 min. The DNA-LipofectAMINE mixture was then diluted with Opti-MEM I re-duced serum medium and added to 90% confluent HEK293 cells plated on 13.5 x 20.1-mm glass coverslips using 0.625 μg of DNA (for measurement of Ca2+) or in 100 mm Petri dishes using 3.75 μg of DNA (for obtaining protein for Western analysis). After 5 h of incubation at 37 °C, equivalent amounts of Opti-MEM I reduced serum medium with 20% fetal bovine serum were added to the medium of transfected cells, and the latter was replaced with fresh DMEM with 10% fetal bovine serum at 24 h after transfection. The expressed Ca2+-sensing receptor protein was assayed 48 h after the start of transfection.

**Measurement of Ca2+ by Fluorometry in Cell Populations**—HEK293 cells, which were plated on coverslips and transfected with CaR cDNAs, were transfected for 2 h at room temperature with cDNA (Promega) in 20 μM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 0.1% (w/v) bovine serum albumin, and 0.1% dextrose and washed once at 37 °C for 20–30 min with a buffer solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.1% dextrose, and 0.1% bovine serum albumin). The coverslips were then placed diagonally in a thermostatted quartz cuvette containing the buffer solution, using a modification of the technique employed previously in this laboratory (23). The CaR was activated by multiple additions of an agonist in incremental doses to reach the desired concentrations. Excitation monochrometers were centered at 340 and 380 nm with emission light collected at 510 ± 40 nm through a wide-band emission filter. The ratio of emitted light intensity as a function of excitation wavelength was plotted as described previously (23) For PKC activation, the cells were preincubated with the PKC activators (PMA, mezeiren, or (−)-indolactam V) for 1–2 min. For PKC inhibition, the cells were preincubated with staurosporine for 30 min. To measure transient Ca2+ responses elicited by neomycin, the buffer solution was devoid of MgCl2, CaCl2, and bovine serum albumin, and 1 mM EGTA was added at the beginning of the experiment. To evaluate the activities of the wild type and mutant receptors, the cumulative Ca2+ response at a given concentration of the agonist was determined using the following method. If the peak increases in Ca2+, are P1, P2, P3... Pn at concentrations of the agonist in the bath solution corresponding to C1, C2, C3... Cn, which were achieved by incremental additions of the agonist, the cumulative Ca2+ response (Rn) at any given agonist concentration (Cn) is defined as the sum, P1 + P2 + P3 +... + Pn. The responsiveness of the wild type and mutant receptors to agonists were compared by determining both EC50 values and the maximal responses of the respective CaRs. EC50 has been defined as the effective concentration of an agonist giving half of the maximal Ca2+ response, and was determined by plotting the concentration-response curve. The cumulative maximal Ca2+ response has been defined as the cumulative Ca2+ response at the highest agonist concentration achieved by the last addition.

**Statistical Analysis**—The mean EC50 for the wild type or each mutant receptor in response to increasing concentrations of CaR agonists was calculated from the EC50 values for all of the individual experiments and is expressed with the S.E. as the index of dispersion. Comparisons of the EC50 values were performed using analysis of variance and Duncan’s multiple comparison, a value of p < 0.05 was considered to indicate a statistically significant difference.

**Crude Plasma Membrane Preparations from Transfected HEK293 Cells**—Crude plasma membrane preparations were isolated from HEK293 cells transiently transfected with the wild type or mutant receptors by differential speed centrifugation as described by Sun et al. (25). Confluent cultured cells in 100-mm culture plates were rinsed twice with phosphate-buffered saline and treated with 0.02% EDTA in phosphate-buffered saline at 37 °C for 5 min. The detached cells were pelleted and suspended in 300 μl of homogenization buffer: 50 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose, 2 mM EDTA, and a mixture of protease inhibitors (83 μg/ml aprotinin, 30 μg/ml leupeptin, 1 mg/ml Pefabloc, 50 μg/ml calpain inhibitor, 50 μg/ml bestatin, and 5 μg/ml pepstatin (Boehring Mannheim)). Then the cells were homogenized with 15 strokes of a motor-driven Teflon pestle in a tightly fitting glass tube. The homogenate was sedimented at 18,800 g for 20 min to remove nuclei and mitochondria. The supernatant was subsequently sedimented at 43,500 × g for 20 min to pellet the plasma membranes, and the resultant pellet was solubilized with 1% Triton X-100. All steps were carried out on ice.

**Western Analysis of Plasma Membrane Proteins**—After determination of protein concentrations in the crude plasma membrane preparations using the Pierce BCA protein assay, an appropriate amount of membrane protein (4 μg) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (26). The proteins on the gel were subsequently electrotransferred to a nitrocellulose membrane. After being blocked with 5% milk, the blot was incubated with a previously characterized...
primary anti-CaR antibody (4641) (20) and then with a secondary, goat antirabbit antibody conjugated to horseradish peroxidase (Sigma, diluted 1:500). The CaR-sensing receptor protein was detected with an ECL system (Amersham Pharmacia Biotech).

Immunoprecipitation of Flag-tagged CaRs—HEK293 cells transiently transfected with receptors were rinsed twice with phosphate-buffered saline and solubilized with 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, pH 7.4, 2 mM EDTA, 1 mM EGTA, protease inhibitors, including 83 μg/ml aprotinin, 30 μg/ml leupeptin, 1 mg/ml Pefabloc, 50 μg/ml calpain inhibitor, 50 μg/ml bestatin, and 5 μg/ml pepstatin (2× immunoprecipitation buffer), at room temperature. Insoluble materials were removed by centrifuging the cell lysates at 15,000 rpm for 15 min at 4 °C. The supernatants were collected as total cell lysates. The protein concentration was determined using the Pierce BCA protein assay. To a microcentrifuge tube, 5 g of protein were added. The mixture was incubated at 4 °C for 1 h. To the mixture was then added 50 μl of 10% protein A-agarose (Life Technologies, Inc.) for an additional 3-h incubation at 4 °C. The supernatants were collected as total cell lysates. The protein concentration was determined using the Pierce BCA protein assay. To a microcentrifuge tube, 5 μg of monoclonal anti-Flag M2 antibody (VWR Scientific), 400 μl of H2O, 500 μl of 2× immunoprecipitation buffer, and 100 μl of total lysate containing 500 μg of protein were added. The mixture was incubated at 4 °C for 1 h. To the mixture was added 5 μl of an alkaline phosphatase-conjugated, anti-mouse IgG. The incubation was continued for an additional 30 min at 4 °C. To the mixture was then added 50 μl of 10% protein A-agarose (Life Technologies, Inc.) for an additional 3-h incubation at 4 °C. The immunoprecipitates were washed three times with 1× immunoprecipitation buffer and twice with phosphate-buffered saline containing protease inhibitors as described above. After one additional wash with 50 μl of PKC assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl2) purchased from Upstate Biotechnology, the samples were ready for in vitro phosphorylation.

In vitro phosphorylation of CaRs—The samples were phosphorylated with 80 ng of PKC (Upstate Biotechnology) in 50 μl of 20 mM MOPS, pH 7.2, containing 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl2, and 100 μM [γ-32P]ATP, 15 mM MgCl2, 0.1 mg/ml phosphatidylserine, 0.01 mg/ml diglyceride, 0.4 μM protein kinase A inhibitor peptide, 4 μM compound R24571 (an inhibitor for calmodulin-dependent kinases). The samples were incubated for 10 min at 32 °C and washed three times with a washing buffer (10 mM Tris-HCl, pH 7.4, 75 mM NaF, 20 mM β-glycerophosphate, 0.1% Triton X-100). The pellet was extracted with 45 μl of 2× SDS sample buffer at 65 °C for 30 min. One-third of the eluted sample was subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. Phosphorylation

FIG. 1. Effect of PMA on Ca2+- and neomycin-elicited Ca2+ responses of the CaR. Changes in the emission ratio (340/380 excitation) were measured to assess Ca2+-evoked Ca2+ responses in fura-2-loaded HEK293 cells transfected with the CaR, which are directly proportional to changes in Ca2+. A and B, the tracings shown are representative of the patterns of the Ca2+-evoked Ca2+ responses seen in cells pretreated with Me2SO (A) and 100 nM PMA (B). The addition of Me2SO or PMA is marked with the first arrowhead on the left. At each subsequent arrowhead, the concentration of Ca2+ was increased first in 1 mM increments to 10, 15, and 20 mM as indicated. C and D, Ca2+ influx was prevented by stimulating the cells with neomycin in Ca2+-free solution. The tracings shown are representative of the patterns of the neomycin-evoked Ca2+ responses seen in multiple experiments in cells mock-treated (C) or treated with 1 μM PMA (D) for 1–3 min. At each arrowhead, the concentration of neomycin was increased in 50 μM increments to 700 μM as indicated. The mean EC50 values are reported under “Results.”

X-100. The pellet was extracted with 45 μl of 2× SDS sample buffer at 65 °C for 30 min. One-third of the eluted sample was subjected to SDSPAGE and electrotransferred to a nitrocellulose membrane. Phosphorylation-
RESULTS

To examine the effects of PKC activators on the CaR, transiently transfected HEK293 cells were treated for 1–3 min with the vehicle (Me2SO), PKC activators (PMA, mezerein, or (-)-indolactam V), or an inactive phorbol analogue (4a-phorbol 12,13-didecanoate, used as a negative control) prior to activation of the CaR by elevating Ca2+. The activity of the receptor was evaluated by measuring EC50 and maximal cumulative response (see under “Experimental Procedures” for definition). Cells treated with Me2SO or 1 μM inactive phorbol derivative responded to increasing concentrations of Ca2+ in a manner similar to untreated cells, with EC50 values of 4.1 ± 0.1 (n = 7), 4.0 ± 0.1 (n = 8), and 4.0 ± 0.1 (n = 34) mM, respectively, without significant differences (p ≥ 0.05). A representative tracing of the control Ca2+-responses is shown in Fig. 1A. In cells treated with 100 nM PMA, the Ca2+-responses at low Ca2+ concentrations (1.5–4.5 mM) were markedly attenuated (Fig. 1B). At higher concentrations of Ca2+ (5.5 mM and above), however, the Ca2+-elicited increases in Ca2+ were similar to those of control cells. As a result, the cumulative maximal Ca2+-response to elevated Ca2+ was reduced to 41% of the control by PMA, with a significant increase in EC50(Ca2+) to 5.0 ± 0.1 mM (n = 25) (p ≥ 0.05). Additional, structurally unrelated PKC activators, such as 1 μM mezerein, and 500 mM (-)-indolactam V had essentially identical inhibitory effects on CaR-elicited Ca2+-responses (Fig. 2).

Because release of Ca2+ from intracellular stores as well as Ca2+ influx could contribute to increases in Ca2+, when Ca2+- is used to activate the CaR, it is essential to use other CaR agonists, such as neomycin, to determine the impact of PKC activators on CaR-evoked mobilization of intracellular Ca2+ in the absence of Ca2+-. The CaR was activated by neomycin in the absence of Ca2+- and Mg2+-, as well as in the presence of 1 mM EGTA. Therefore, the neomycin-elicited Ca2+-responses in cells transfected with the CaR (Fig. 1C) were solely the result of release of Ca2+ from intracellular stores, and these responses were not present in cells mock-transfected with vector alone (data not shown). Neomycin-elicited Ca2+-responses were substantially attenuated by pretreatment with PMA (1 μM) (Fig. 1D) at all concentrations of neomycin tested, and the maximal cumulative Ca2+-response was reduced to 25% of the control. PMA treatment increased the EC50(neomycin) of the CaR from 298 ± 9 μM (n = 8) to 405 ± 22 μM (n = 8). The marked difference in the maximal cumulative responses in PMA-treated cells stimulated with Ca2+- versus neomycin in the absence of Ca2+ (64 and 25%, respectively) suggested that calcium influx stimulated by CaR agonists might be less affected by PMA than calcium mobilization from intracellular stores.

To evaluate the effect of PMA on high Ca2+-evoked sustained increases in Ca2+, as an indirect assessment of Ca2+ influx, Ca2+-elicited transient increases in Ca2+, were permitted to fall for a longer period of time (100 s in Fig. 3 versus 25 s in Fig. 1) after each addition of Ca2+. The sustained responses observed in this experiment were defined as the increased levels of Ca2+ that remained at 100 s after each incremental addition of agonist, and these responses reflect the new steady states, in which influx and efflux of Ca2+ are nearly equal. As shown in Fig. 3, PMA markedly reduced the transient Ca2+-responses at low Ca2+ concentrations (1.5–3.5 mM), similar to the earlier observations made in Fig. 1, A and B. Thus the cumulative transient Ca2+-responses were markedly affected by PMA (Fig. 4A). In contrast, the sustained Ca2+-responses were similar in control and PMA-treated cells (Fig. 4B). In cells mock-transfected with vector alone, there was also a gradual Ca2+-increase. However, at least 60% of this increase resulted
from leakage of fura-2 over the ~20-min time course of these experiments, which was marginally affected by PMA (Fig. 3, C and D, and Fig. 4). Although the transfection efficiency was less than 25%, the sustained \( \text{Ca}^{2+}\text{i} \) responses in the receptor-transfected cells were significantly higher than those in vector-transfected cells.

In order to demonstrate further that the effect of PKC activators on the function of the CaR was mediated by PKC, we examined the effect of a PKC inhibitor, staurosporine, on CaR-evoked \( \text{Ca}^{2+}\text{i} \) responses (Fig. 5). Pretreatment of CaR-transfected HEK293 cells with 1 \( \mu \text{M} \) staurosporine for 30 min significantly reduced the EC_{50} \( \text{Ca}^{2+}\text{a} \) from 4.0 ± 0.1 mM (n = 34; Table I) to 2.9 ± 0.1 mM (n = 22; Table I) (p ≤ 0.05). In addition, the pretreatment prevented the inhibitory effects of PMA (Fig. 5) and other PKC activators (data not shown) on the \( \text{Ca}^{2+}\text{i} \) responses to the same extent, further supporting the conclusion that the effect of PKC activators on CaR-dependent changes in \( \text{Ca}^{2+}\text{i} \) is through activation of PKC.

We next determined which residues are involved in PKC-mediated regulation of the CaR by performing site-directed mutagenesis on the five predicted PKC sites. One of these sites, Thr646, is located in the first intracellular loop; another one, Ser794, is in the third intracellular loop; and the remainder (Thr888, Ser895, and Ser915) are in the cytoplasmic tail. The expression of these mutant receptors were then examined by Western analysis, as described previously (20). After transient transfection, crude plasma membrane proteins were isolated and subjected to reduced 4–12% SDS-PAGE, and the CaRs were detected using a specific anti-CaR antibody. In Fig. 6, the two bands between 140 and 200 kDa are monomeric forms of the CaR; the bands above 200 kDa are also specific for the CaR and are not present in the vector-transfected cells, as shown previously (20) (see also Fig. 9). None of the mutations substantially altered the expression level of the receptor.
**Ca$$^{2+}$$, Dynamics in Cells Transfected with the CaR**

### Table I

| Mutation          | EC$_{50}$ [Ca$$^{2+}$$] Control | EC$_{50}$ [Ca$$^{2+}$$] PMA$^a$ | EC$_{50}$ [Ca$$^{2+}$$] Staurosporine$^a$ | EC$_{50}$ [Ca$$^{2+}$$] Staurosporine/PMA$^a$ |
|-------------------|---------------------------------|---------------------------------|------------------------------------------|-----------------------------------------------|
| Wild type         | 4.0 ± 0.1 (34)                  | 5.0 ± 0.1 (25)                  | 2.9 ± 0.1 (22)                           | 2.9 ± 0.1 (8)                                 |
| T646V             | 4.0 ± 0.1 (8)                   | 5.3 ± 0.1 (4)                  | 2.8 ± 0.2 (4)                            |                                               |
| S794A             | 4.0 ± 0.2 (6)                   | 4.9 ± 0.1 (4)                  | 2.8 ± 0.2 (4)                            |                                               |
| T888V             | 2.9 ± 0.1 (5)$^b$              | 3.3 ± 0.1 (5)                  | 2.3 ± 0.1 (5)                            |                                               |
| S895A             | 3.6 ± 0.1 (10)$^b$             | 4.5 ± 0.1 (4)                  | 2.4 ± 0.1 (4)                            |                                               |
| S915A             | 3.6 ± 0.1 (11)$^b$             | 4.9 ± 0.0 (4)                  | 2.6 ± 0.1 (4)                            |                                               |
| T888V/S895A       | 3.0 ± 0.1 (11)$^b$             | 3.2 ± 0.1 (10)                 | 2.2 ± 0.1 (11)                           |                                               |
| T888V/S915A       | 3.0 ± 0.1 (7)$^b$              | 3.5 ± 0.1 (4)                  | 2.1 ± 0.1 (4)                            |                                               |
| S895A/S915A       | 3.4 ± 0.1 (10)$^b$             | 4.5 ± 0.1 (13)                 | 2.4 ± 0.1 (12)                           |                                               |
| Triple            | 2.6 ± 0.1 (22)$^b$             | 2.9 ± 0.1 (16)                 | 2.2 ± 0.1 (13)                           | 2.2 ± 0.1 (6)                                 |
| Quintuple         | 2.6 ± 0.1 (12)$^b$             | 3.1 ± 0.1 (7)                  | 2.4 ± 0.1 (5)                            | 2.2 ± 0.1 (5)                                 |

$^a$ Significantly ($p \leq 0.05$) different from controls.

$^b$ Significantly ($p \leq 0.05$) different from the wild type control.

**Fig. 6. Western analysis to assess expression of the wild type and mutant receptors.** Crude plasma membrane proteins (4 μg) isolated from CaR-transfected HEK293 cells were subjected to a 4–12% gradient SDS-PAGE in the order (from left to right): wild type CaR, T646V, S794A, T888V, S895A, S915A, T888V/S895A, T888V/S915A, S895A/S915A, triply mutated CaR (T888V/S895A/S915A), and quintuply mutated CaR. The receptor proteins were detected with a specific anti-CaR antiserum, 4641, as described under “Experimental Procedures.” The blot shown is a representative of the pattern seen in two protein preparations from two independent transfections.

*Significantly ($p \leq 0.05$) different from controls.

*Significantly ($p \leq 0.05$) different from the wild type control.

for 1–3 min. The patterns of the Ca$$^{2+}$$ responses for receptors with the single point mutations, T646V, S794A, S895A, or S915A (data not shown), or with the double mutation, S895A/S915A (data not shown), were similar to that of the wild type receptor (Fig. 1B). PMA markedly attenuated low Ca$$^{2+},$$-elicited Ca$$^{2+},$$ responses mediated by these mutant receptors. As shown in Fig. 7 and Table I, the EC$_{50}$[Ca$$^{2+}$$] values for these five mutant receptors were significantly increased by PMA in a manner similar to that observed with the wild type receptor. In sharp contrast, PMA produced only small increases in the EC$_{50}$[Ca$$^{2+}$$] values of all of the mutant receptors containing T888V. The receptor with T888V alone showed some decrease in its Ca$$^{2+},$$ responses at 1.5, 2.5, and 3.5 mM Ca$$^{2+},$$ (data not shown). Receptors with one more mutation in addition to Thr888, such as T888V/S895A and T888V/S915A, did not show any alterations in the pattern of the Ca$$^{2+},$$ responses observed with the mutant receptor with T888V alone (data not shown). However, receptors with two or more additional mutations, such as the triply mutated (T888V/S895A/S915A) and quintuply mutated CaRs, exhibited markedly reduced inhibitory effects of PMA, only showing some decrease in the Ca$$^{2+},$$ response at 1.5 mM Ca$$^{2+},$$ (data not shown). Thus, the effect of PKC activation by PMA on CaR-mediated increases in Ca$$^{2+},$$ was mostly mediated by one PKC site, Thr888.

To demonstrate that the effect of staurosporine on the wild type CaR was, at least in part, the result of prevention of PKC-induced phosphorylation of the receptor, we examined the effect of staurosporine on the mutant receptors carrying PKC site mutations. As shown in Fig. 7 and Table I, staurosporine reduced EC$_{50}$[Ca$$^{2+}$$] values of all of the mutant receptors to varying extents. The receptors with the most critical PKC site mutated, T888V, which showed the smallest increases in their Ca$$^{2+},$$ responses at 1.5, 2.5, and 3.5 mM Ca$$^{2+},$$ (data not shown). Thus, the effect of PKC activation by PMA on CaR-mediated increases in Ca$$^{2+},$$ was mostly mediated by one PKC site, Thr888.

In order to determine which PKC sites are responsible for the inhibitory effects of PKC activators on CaR agonist-evoked increases in Ca$$^{2+},$$ cells transfected with mutant receptors were pretreated with one of the PKC activators, 100 nM PMA,
had been transfected with the wild type and mutant receptors, including the doubly mutated (S895A/S915A) and the quadruply mutated (T646V/S794A/S885A/S915A) receptors, which preserved the crucial PKC site, Thr888. PMA substantially diminished the CaR responses and markedly increased the EC50(neomycin) values of the receptors (wild type receptor, Fig. 1D; data not shown for the mutant receptors). In contrast, PMA had much reduced effects on the CaR responses of cells transfected with mutant receptors containing T888V. The EC50(neomycin) of the mutant CaR harboring T888V, for instance, was not significantly affected by PMA (i.e. 202 ± 7 µM (n = 10) before versus 207 ± 13 µM (n = 8) after the addition of PMA; p ≤ 0.05). Likewise, PMA had no significant effect on the EC50(neomycin) of the quintuply mutated receptor (146 ± 7 µM (n = 8) before versus 137 ± 7 µM (n = 8) after the addition of PMA; p ≤ 0.05). Representative tracings of CaR responses are shown in Fig. 8. Although the maximal cumulative responses of the T888V and quintuply mutated receptors in the presence of PMA were reduced to about 50 and 60% of the control, respectively, the CaR-specific bands at their respective positions (Fig. 9, lanes 1-4) were similar for all of the CaR receptors when we detected with anti-CaR antibody, 4641, and are absent in Fig. 9B, lane 5, i.e. the vector control. Therefore, in conclusion, we have established that Thr888 is the major site of the CaR that is phosphorylated by PKC in vitro. Moreover, this site mediates the PKC-induced uncoupling of the receptor from release of intracellular Ca2+ stores.

**DISCUSSION**

Ca2+, is the main physiological regulator of PTH secretion through actions on its own cell surface receptor, i.e. the CaR (27). Increases in the concentration of Ca2+ elicit rapid, transient Ca2+ responses followed by sustained increases in Ca2+ in parathyroid cells and CaR-transfected HEK293 cells. Previous studies and our present results show that both activation and inhibition of PKC have profound effects on Ca2+ -elicited Ca2+ responses in these cells. In the present study, we found that these effects can be largely eliminated by mutating Thr888 in the CaR. These results also suggest that this site may have been partially phosphorylated by PKC under our standard experimental conditions; therefore, the activity of the CaR can potentially be modulated by either activating or inhibiting PKC in vivo, in agreement with previous studies in bovine parathyroid cells (6).

Activation of PKC by PMA mainly blocked the CaR-mediated Ca2+ responses resulting from mobilization of Ca2+ from intracellular stores in cells transfected with the wild type receptor or with mutant CaRs in which the PKC site, Thr888, was preserved. This PMA-induced inhibition of CaR-mediated Ca2+ release is particularly apparent with the use of an alternative CaR agonist, neomycin, in the absence of extracellular Ca2+, when uptake of Ca2+, was totally eliminated (Fig. 1D). We were able to block substantially the inhibitory effect of PKC activation on CaR-induced release of Ca2+ stores by mutating threonine 888. Therefore, PKC-mediated phosphorylation of the CaR at threonine 888 markedly uncouples the receptor from release of Ca2+ from its intracellular stores.

Phosphorylation at position 888 in all of the CaR receptors phosphorylation had little impact on Ca2+ -elicited Ca2+ responses at 5.5 mM or higher concentrations of Ca2+ (e.g. Fig. 1, A and B), even though PMA markedly inhibited neomycin-elicited mobilization of Ca2+ at all concentrations tested in the absence of Ca2+. Thus, it is reasonable to assume that high Ca2+ -elicited Ca2+ responses in the presence of PMA largely result from
Ca\(^{2+}\) influx (Fig. 1B). Consistent with the hypothesis that PMA has little or no impact on high Ca\(^{2+}\)-stimulated, CaR-mediated Ca\(^{2+}\) influx, PMA had no effect on CaR activation-dependent sustained Ca\(^{2+}\) increases, which are presumably maintained by CaR-induced increases in Ca\(^{2+}\) influx and/or decreases in Ca\(^{2+}\) efflux. Nevertheless, it is premature to conclude that PKC phosphorylation at position 888 has no impact on CaR-stimulated Ca\(^{2+}\) influx, because it is hard to distinguish between the contributions of extracellular and intracellular sources of calcium to increases in Ca\(^{2+}\), when extracellular calcium is employed as an agonist for the CaR. To elucidate fully the effects of PMA, if any, on CaR-activation dependent Ca\(^{2+}\) influx, further studies are needed to measure directly Ca\(^{2+}\) influx.

CaR activation-dependent Ca\(^{2+}\) influx may be mediated, in part, by Ca\(^{2+}\)-permeable, nonselective cation channels that we have observed in both parathyroid cells (28) and HEK293 cells (29). CaR agonists (neomycin and Ca\(^{2+}\)\(_o\)) significantly increase the probability of channel opening in HEK293 cells stably transfected with the CaR but not in nontransfected HEK293 cells that do not express the CaR (29). Thus, the enhanced activity of Ca\(^{2+}\)-permeable nonselective cation channels in CaR-transfected HEK293 cells could contribute to the sustained increases in Ca\(^{2+}\) in the presence of CaR agonists. These nonselective cation channels are also thought to contribute to influx of Ca\(^{2+}\)\(_o\) into hippocampal neurons and to regulation of their excitation (30, 31).

In PMA-treated cells transfected with the wild type CaR, there was still some residual Ca\(^{2+}\) response (~25%) to neomycin, even in the total absence of Ca\(^{2+}\) influx. A substantially higher concentration of neomycin (350 versus 150 \(\mu\)M in the absence of PMA) was required to elicit the initial response, consistent with observations made earlier in this laboratory (6). That is, activators of PKC inhibited by 50–60% the high Ca\(^{2+}\)-stimulated generation of inositol phosphates in CaR-expressing bovine parathyroid cells and reduced inositol trisphosphate levels at low Ca\(^{2+}\)\(_o\) presumably by reducing turnover of phosphoinositides by phospholipase C. It is possible that PMA selectively uncouples the receptor from one subtype of G-protein but not another, both of which activate phospholipase C. In rat portal vein myocytes, \(G_{q}\) and \(G_{11}\) have been

**Fig. 8.** Mutation of one PKC site (T888V) blocks the PMA effect on neomycin-elicited Ca\(^{2+}\) store release. HEK293 cells were transfected with a CaR containing T888V (A and B) or five mutated PKC sites (C and D). Neomycin-evoked Ca\(^{2+}\) increases in the transfected cells were measured as in Fig. 1, C and D. The tracings shown are representative of the patterns of the neomycin-evoked Ca\(^{2+}\) responses seen in cells mock-treated (A and C) or treated for 1–3 min with 1 \(\mu\)M PMA (B and D). At each arrowhead, the concentration of neomycin was increased in 50 \(\mu\)M increments to 700 \(\mu\)M as indicated. The mean EC\(_{50}\)[neomycin] values are reported under “Results.”

**Fig. 9.** In vitro phosphorylation of the wild type and mutant CaRs. HEK293 cells transfected with Flag-tagged CaRs or the vector were solubilized with Triton X-100 and IGEPA CA-630 (see under “Experimental Procedures”), and the CaRs were immunoprecipitated with anti-Flag antibody before phosphorylation with a mixture of PKCa, -\(\beta\), and -\(\gamma\). The samples were resolved on a 2.2–8.2% gradient SDS-PAGE. The phosphorylated proteins were visualized by autoradiography (A), and the CaR-immunoreactive species were detected by anti-CaR antibody (B). wt, wild type. The result shown is a representative of two phosphorylation experiments.
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shown to have distinct functions in coupling \(\alpha_1\)-adrenergceptors to Ca\(^{2+}\) release and Ca\(^{2+}\) entry (32). In this system, it appeared that G\(_i\) activated hydrolis of phosphatidylinositol 4,5-bisphosphate with an attendant release of Ca\(^{2+}\) from inositol trisphosphate-sensitive intracellular stores, whereas G\(_{11}\) enhanced Ca\(^{2+}\) influx. By analogy, it is possible that phosphorylation of threonine at amino acid position 888 uncouples the Ca\(^{2+}\) from G\(_i\) but not G\(_{11}\), thereby largely inhibiting release of Ca\(^{2+}\) from intracellular stores but not Ca\(^{2+}\) influx.

When we mutated all five predicted PKC sites in the CaR, there were still some residual effects of the PKC activator PMA to Ca\(^{2+}\) influx. Alternatively, PMA may reduce store capacity as has been shown on other components in the signal transduction pathway. Alternatively, PMA may reduce store capacity as has been shown on other components in the signal transduction pathway. Alternatively, PMA may reduce store capacity as has been shown on other components in the signal transduction pathway.

In summary, we have demonstrated that PKC modulation of CaR-mediated Ca\(^{2+}\) responses is primarily mediated by Thr888, which can be phosphorylated by PKC in vitro. Phosphorylation at Thr888 inhibits most of the agonist-induced increases in Ca\(^{2+}\) due to release from intracellular stores. In parathyroid cells, we postulate that the phospholipase C/inositol trisphosphate pathway, leading to release of Ca\(^{2+}\) from stores and/or other downstream effects, is associated with PKC regulation of PTH secretion. In addition, regulation of PKC activity in vivo provides a means of modulating the function of the CaR and, ultimately, calcium homeostasis.

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