Protein Kinase C (PKC) Phosphorylation of the $\text{Ca}^{2+}$-sensing Receptor (CaR) Modulates Functional Interaction of G Proteins with the CaR Cytoplasmic Tail

Yong-Feng Jiang, Zaixiang Zhang, Olga Kifor, Charles R. Lane, Stephen J. Quinn, and Mei Bai

From the Endocrine-Hypertension Division, Department of Medicine, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02115

The extracellular calcium ($\text{Ca}^{2+}$)-sensing receptor (CaR) activates $\text{Ca}^{2+}$ influx independent of the release of intracellular $\text{Ca}^{2+}$ stores. The latter can be negatively regulated by protein kinase C (PKC) through phosphorylation of Thr-888 of the CaR. In this study, we substituted Thr-888 with various amino acid residues or a stop codon to understand how PKC phosphorylation of the CaR inhibits receptor-mediated release of intracellular $\text{Ca}^{2+}$ stores. Substitutions of Thr-888 with hydrophobic and hydrophilic amino acid residues had various effects on CaR-mediated release of intracellular $\text{Ca}^{2+}$ stores as well as activation of $\text{Ca}^{2+}$ influx. Several point mutations, such as T888D, had marked negative effects on CaR-mediated release of intracellular $\text{Ca}^{2+}$ stores but not on phorbol myristate acetate-insensitive activation of $\text{Ca}^{2+}$ influx. Presumably, the negatively charged aspartate mimics phospho-threonine. Interestingly, truncating the receptor at 888 had an even more pronounced negative effect on CaR-elicited release of intracellular $\text{Ca}^{2+}$ stores but not on phorbol myristate acetate-insensitive activation of $\text{Ca}^{2+}$ influx. Therefore, truncation at position 888 of the CaR affects the activity of the receptor in a manner that resembles PKC phosphorylation of the CaR. This in turn suggests that PKC phosphorylation of the CaR prevents G protein subtypes from interacting with the region of the receptor critical for releasing $\text{Ca}^{2+}$ stores, which is missing in the truncated receptor.

Extracellular calcium concentration ($\text{Ca}^{2+}$) is tightly regulated by the interactions of several hormones (e.g. parathyroid hormone, vitamin D, and calcitonin) and organ systems (i.e. parathyroid gland, kidney, bone, and intestine) (1). Parathyroid cells respond to changes in $\text{Ca}^{2+}$ by oppositely directed alterations in parathyroid hormone secretion through the CaR (13, 14). Likewise, PKC may contribute to age-related changes in the regulation of parathyroid hormone secretion by $\text{Ca}^{2+}$ in rats (15). Therefore, stimulus-secretion coupling in parathyroid cells can be modulated by PKC. We recently showed that some of this modulation occurs at the receptor level (16).

The human homologue of the CaR has five putative PKC phosphorylation sites in its intracellular domains. An alteration in the coupling of the CaR to secondary signaling pathways has been implicated as one of mechanisms by which PKC exerts negative effects on the function of the receptor (16). In particular, PKC phosphorylation of Thr-888 inhibits CaR-mediated increases in cytosolic calcium ($\text{Ca}^{2+}$) via mobilization of intracellular $\text{Ca}^{2+}$ stores. In this study, we introduced various mutations at position 888 and examined whether any of these mutations mimic or block the action of PKC activators on CaR-mediated PLC signaling pathways. We found that truncating the CaR at 888 impairs the function of the receptor in a similar way as activating cellular PKC.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis utilized the approach described by Kunkel (17) to produce mutated receptors in which the threonine residue at position 888, one of the five predicted PKC phosphorylation sites in the cytoplasmic tail of the human CaR, was mutated to various amino acid residues or a stop codon. The dut-1 ung-1 strain of Escherichia coli, CJ236, was transformed separately with mutagenesis cassette 6, as described previously (18). Uracil-containing, single-stranded DNA was produced by infecting the cells with the helper phage VCSM13 (Stratagene, La Jolla, CA). The single-stranded DNA was then annealed to a mutagenesis primer that contained the desired nucleotide change encoding a single-point mutation flanked on both sides by wild-type sequences. The primer was subsequently extended around the entire single-stranded DNA and ligated to generate closed circular heteroduplex DNA. DH5α competent cells were transformed with these DNA heteroduplexes, and incorporation of the desired mutation was confirmed by sequencing the entire cassette. The resultant mutated cassette 6 containing the desired mutation was doubly digested with XhoI and XbaI and cloned into the full-length of the receptor in pcDNA3.

Transient Expression of CaRs in HEK293—CaR DNA was prepared with the Mβase plasmid kit (Qiagen). LipofectAMINE (Invitrogen) was employed as a DNA carrier for transfection. The HEK293 cells used for transient transfection were provided by NPS Pharmaceuticals (Salt Lake City, UT) and were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Hyclone). The DNA-liposome complex was prepared by mixing DNA and LipofectAMINE in OPTI-MEM I reduced serum medium (Invitrogen) and incubating the mixture at room temperature for 30 min. The DNA-LipofectAMINE mixture was then diluted with OPTI-MEM I reduced serum medium and added to confluent HEK293 cells plated on 13.5 × 20.1 mm glass coverslips using 2.5 μg of DNA. After the cells were incubated for 5 h at 37 °C, an equivalent amount of OPTI-MEM I reduced serum medium with 20% fetal bovine serum was added to the medium overlaying the transfected cells, and the latter was replaced with fresh...
Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 24 h after transfection. The expression of CaR-sensing receptor protein was assayed 48 h after the start of transfection.

**Detection of Expressed CaR on the Cell Surface**—Before whole cell lysates were prepared, intact HEK293 cells transiently transfected with FLAG-tagged CaR were labeled with 1 mM ImmunoPure was assayed 48 h after the start of transfection. The expressed CaR was immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma). The coverslips were then placed diagonally in a thermostatted quartz cuvette that contained the buffer solution using a chemiluminescence (ECL) system (PerkinElmer Life Sciences).

**TABLE I**

| Mutants      | EC$_{50}$ [Ca$^{2+}$]$^a$ | Max response$^a$ | EC$_{50}$ [Spermine]$^a$ | Max response$^a$ |
|--------------|---------------------------|------------------|---------------------------|------------------|
|              | µM                        | %                | µM                        | %                |
| WT           | 3.0 ± 0.1 (22)            | 3.5 ± 0.1 (13)$^d$ | 176.0 ± 3.9               | 89.5 ± 2.1$^d$   |
| T888V        | 2.4 ± 0.1 (10)$^d$        | 2.4 ± 0.1 (6)    | 134.3 ± 5.5               | 101.7 ± 4.0$^d$  |
| T888Y        | 2.4 ± 0.1 (5)$^d$         | ND               | 141.2 ± 2.0               | ND               |
| T888W        | 2.2 ± 0.1 (5)$^d$         | ND               | 135.2 ± 4.8               | ND               |
| T888A        | 2.2 ± 0.1 (5)$^d$         | ND               | 135.2 ± 9.1               | ND               |
| T888Q        | 2.8 ± 0.1 (5)$^d$         | ND               | 161.2 ± 12.1              | ND               |
| T888G        | 3.0 ± 0.1 (5)$^d$         | ND               | 152.1 ± 8.3               | ND               |
| T888S        | 3.4 ± 0.1 (5)$^d$         | ND               | 176.7 ± 4.5               | ND               |
| T888K        | 2.9 ± 0.1 (13)$^d$        | 3.0 ± 0.1 (5)    | 109.4 ± 4.4               | 105.7 ± 6.5$^d$  |
| T888E        | 3.3 ± 0.1 (14)$^d$        | 3.2 ± 0.1 (8)    | 150.0 ± 6.0               | 95.1 ± 3.6$^d$   |
| T888D        | 3.4 ± 0.1 (10)$^d$        | 3.5 ± 0.1 (8)    | 135.1 ± 10.4              | 94.0 ± 6.8$^d$   |
| T888Stop     | 3.4 ± 0.1 (12)$^d$        | 3.3 ± 0.1 (8)    | 92.3 ± 3.0                | 86.8 ± 5.2$^d$   |

$^a$ EC$_{50}$a are means ± S.E. The number of the experiments is indicated in the parentheses.

$^b$ Values are significantly different from the values of their corresponding controls (p < 0.05).

$^c$ EC$_{50}$s of the mutant receptors are significantly different from those of the wild-type receptor.

$^d$ ND, not determined.

**RESULTS**

The impact of various substitutions of Thr-888 on the function of the CaR was examined by stimulating the mutant receptors in transiently transfected HEK293 cells by Ca$^{2+}$ for a full CaR response or by spermine in the absence of Ca$^{2+}$ to avoid Ca$^{2+}$ influx. The activities of mutant receptors were evaluated initially by measuring Ca$^{2+}$ responses. As summarized in Table I, the substitutions of hydrophobic amino acid residues for Thr-888, including the previously reported T888V, reduced EC$_{50}$[Ca$^{2+}$], significantly, whereas the substitutions of negatively charged amino acid residues or glycine increased EC$_{50}$[Ca$^{2+}$]. The other substitutions, such as Asn, Gln, and Lys, did not change EC$_{50}$[Ca$^{2+}$], significantly. The patterns of the Ca$^{2+}$ responses of all the mutant receptors were somewhat different from those of the wild-type receptor (Fig. 1). Among them, T888K and T888D showed little transient responses but did show prominent sustained responses that were indicative of activation of Ca$^{2+}$ influx. In addition to substituting Thr-888 with various amino acid residues, we also truncated the receptor by introducing a stop codon at position 888. We found that T888Stop further blunted transient Ca$^{2+}$ responses with an increased EC$_{50}$[Ca$^{2+}$] and a 50% reduction in its maximal cumulative Ca$^{2+}$ response. In addition, the pattern of the Ca$^{2+}$ responses of T888Stop showed a close resemblance to that of the wild-type receptor in PMA-treated cells (Fig. 1) in which CaR-evoked transient Ca$^{2+}$ increases due to the mobilization of intracellular Ca$^{2+}$ stores were substantially blunted.

Both the release of Ca$^{2+}$ from intracellular stores and activation of Ca$^{2+}$ influx contribute to CaR-mediated increases in Ca$^{2+}$, when Ca$^{2+}$ is used as a CaR agonist. Therefore, we also used an alternative CaR agonist, spermine, to determine the impact of PKC activation on CaR-evoked mobilization of intracellular Ca$^{2+}$ stores. In the following experiment, we stimulated the receptor with spermine in the absence of Ca$^{2+}$ and Mg$^{2+}$. A trace amount of divalent cations was removed by including 1 mM EGTA in the experimental buffer prior to the
addition of spermine. All mutant receptors had significant spermine-elicited Ca\(^{2+}\) responses, but they were still less than those of the wild-type receptor (Table I). Among them, T888Stop had the lowest maximal response, about 25% of that of the wild-type receptor, followed by T888K and T888D, which had maximal responses about 55% of that of the wild-type receptor. Others had maximal responses ranging from 62% to 99% (Table I). These results suggest that mutations at position 888 affect CaR-mediated activation of certain G protein subtypes that induce the release of intracellular Ca\(^{2+}\) stores.

Next, we tested the effect of PMA on various mutants when stimulated by spermine in the absence of Ca\(^{2+}\) and Mg\(^{2+}\).
Consistent with our previous study, the effects of PMA on the CaR were significantly reduced by all the mutations. As shown in Table I, all mutant receptors except T888Stop showed greater maximal responses in the presence of PMA than did the wild-type receptor. Among them, T888W, T888Y, T888Q, and T888G were most resistant to PMA with maximal responses over 2-fold higher than those of the wild-type receptor in cells similarly pretreated with 100 nM PMA. Although T888Stop-mediated responses were insensitive to PMA, the responses in the control conditions were similar to those of the wild-type receptor in cells pretreated with PMA. As shown in Fig. 2, pretreatment of T888Stop-transfected cells with PMA hardly affected the Ca\(^{2+}\) responses elicited by either spermine or Ca\(^{2+}\). Moreover, the resultant response curves of the truncated receptor under all conditions were essentially superimposed on those of the wild-type receptor pretreated with 100 nM PMA, which markedly attenuated CaR-mediated release of Ca\(^{2+}\) from intracellular stores, as shown in our previous study (16). These results suggest that PKC phosphorylation of Thr-888 blocks the respective G proteins from interacting with the region of the receptor C-terminal to Ala-887 and from activating Ca\(^{2+}\) stores.

To determine whether PKC activation exerts its negative effects on signaling components upstream of mobilization of intracellular Ca\(^{2+}\) stores, we determined IP responses of some receptors. IP responses were determined in the transfected cells prelabeled overnight with [\(^{3}\)H]inositol. For the effects of PKC activation, we pretreated the cells with 100 nM PMA for 10 min and subsequently stimulated the cells with either Ca\(^{2+}\) or spermine. Pretreatment of the cells with PMA reduced Ca\(^{2+}\)-elicited IP responses of the wild-type receptor by 14.5% and shifted the response curve to the right by 2–4 mM Ca\(^{2+}\) (Fig. 3A and Table II). Pretreatment of the cells with PMA had a much milder negative effect on IP responses than on Ca\(^{2+}\) responses when the wild-type receptor was stimulated by spermine alone (Fig. 3B and Table II). For instance, at 1 mM spermine (when the IP response approached the maximal value), pretreatment of cells with PMA reduced the response by 99%. This suggests that the major component of the Ca\(^{2+}\)-elicited IP response of the wild-type receptor, about 85% at concentrations of Ca\(^{2+}\) as high as 8–16 mM, is dependent on activation of Ca\(^{2+}\) influx.

Consistent with the hypothesis that PKC-phosphorylated
TABLE II
Effects of PMA and CaR mutations on CaR-mediated accumulation of total IP in response to Ca\(^{2+}\) or spermine

| Spermine (mM)\(^b\) | Control Wt\(^c\) | Control T888Stop | Control T888V |
|-------------------|---------------|----------------|-------------|
|                   | PMA           | PMA            | PMA          |
| 0.5               | 0.30 ± 0.01 (6) | 0.28 ± 0.02 (4) | 0.38 ± 0.03 (4)\(^p\) | 0.29 ± 0.01 (4)\(^p\) | 0.47 ± 0.04 (8)\(^c\) | 0.35 ± 0.03 (8)\(^c\) |
| 2                 | 0.67 ± 0.03 (15) | 0.39 ± 0.04 (8)\(^d\) | 0.36 ± 0.03 (4)\(^p\) | 0.32 ± 0.03 (4)\(^p\) | 1.03 ± 0.05 (10)\(^p\) | 0.69 ± 0.04 (10)\(^p,d\) |
| 4                 | 1.43 ± 0.05 (16) | 0.79 ± 0.04 (8)\(^d\) | 1.01 ± 0.05 (4)\(^p\) | 0.96 ± 0.08 (4)\(^p\) | 1.73 ± 0.11 (10)\(^p\) | 1.58 ± 0.04 (10)\(^p,d\) |
| 8                 | 1.83 ± 0.03 (16) | 1.50 ± 0.04 (10)\(^d\) | 1.33 ± 0.09 (4)\(^p\) | 1.43 ± 0.05 (4)\(^p\) | 2.24 ± 0.09 (10)\(^p\) | 2.06 ± 0.09 (10)\(^p,d\) |
| 16                | 1.98 ± 0.02 (14) | 1.66 ± 0.07 (10)\(^d\) | 1.61 ± 0.04 (4)\(^p\) | 1.68 ± 0.07 (4)\(^p\) | 2.48 ± 0.10 (10)\(^p\) | 2.26 ± 0.09 (10)\(^p,d\) |

| Sperrmine (mM)\(^b\) | Control Wt\(^c\) | Control T888Stop | Control T888V |
|-------------------|---------------|----------------|-------------|
|                   | PMA           | PMA            | PMA          |
| 0                 | 0.32 ± 0.02 (14) | 0.33 ± 0.03 (6) | 0.28 ± 0.01 (4)\(^p\) | 0.31 ± 0.01 (4)\(^p\) | 0.37 ± 0.04 (6) | 0.35 ± 0.07 (6) |
| 0.5               | 0.58 ± 0.03 (14) | 0.33 ± 0.02 (12)\(^d\) | 0.32 ± 0.03 (6)\(^p\) | 0.30 ± 0.02 (4)\(^p\) | 0.82 ± 0.03 (7)\(^p\) | 0.56 ± 0.04 (7)\(^p,d\) |
| 1                 | 0.86 ± 0.03 (14) | 0.39 ± 0.02 (12)\(^d\) | 0.38 ± 0.04 (6)\(^p\) | 0.35 ± 0.01 (4)\(^p\) | 1.09 ± 0.06 (7)\(^p\) | 0.90 ± 0.05 (7)\(^p,d\) |
| 2                 | 1.00 ± 0.01 (14) | 0.53 ± 0.01 (12)\(^d\) | 0.59 ± 0.01 (6)\(^p\) | 0.54 ± 0.03 (4)\(^p\) | 1.35 ± 0.07 (7)\(^p\) | 1.14 ± 0.06 (7)\(^p,d\) |

\(^a\) Wt, wild type.

Values are means ± S.E. and normalized to the maximal IP response of the wild-type receptor elicited by spermine alone in the absence of PMA. The number of the experiments is indicated in the parentheses.

The IP responses of the mutant receptors are significantly different from those of the wild-type receptor in the same experimental conditions \((p \leq 0.05)\).

Values are significantly different from their corresponding controls \((p \leq 0.05)\).

FIG. 4. Total IP responses of T888Stop elicited by spermine (A) or by Ca\(^{2+}\) (B). The cells transfected with T888Stop were stimulated in the presence \((open)\) or absence \((filled)\) of 100 nm PMA. Formation of total IP in cells prelabeled with \(^{3}H\)inositol was measured as described under “Experimental Procedures.” IP responses were normalized to the maximal spermine-elicited response of the wild-type receptor in the absence of PMA and Ca\(^{2+}\), \(\text{mean} \pm \text{S.E.}; n = 4–6\) and listed in Table II. Significance of any differences between points at a given concentration of agonists is given in Table II. [Image of graph]

FIG. 5. Ca\(^{2+}\) influx-dependent IP response of the CaR in cells transfected with the wild-type receptor and T888Stop. The cells transfected with the wild-type receptor \((A)\) or T888Stop \((B)\) were stimulated by spermine in the presence \((open)\) or absence \((filled)\) of 0.5 mm Ca\(^{2+}\). Formation of total IP in cells prelabeled with \(^{3}H\)inositol was measured as described under “Experimental Procedures.” IP response was normalized to the maximal spermine-elicited response of the wild-type receptor in the absence of Cu\(^{2+}\), \(\text{mean} \pm \text{S.E.}; n = 6–10\). At any given dose of spermine, the data points were marked with asterisks if the responses in the presence and absence of 0.5 mm Ca\(^{2+}\) are significantly different \((p \leq 0.05)\). [Image of graph]
CaR blocks the interaction of certain G proteins with structural elements located C-terminally to Ala-887, no significant IP responses were elicited by spermine even in the absence of PMA (Fig. 4 and Table II). In contrast, Ca\(^{2+}\)/H\(_{11001}\)-induced IP responses of T888Stop were substantial but insensitive to PMA (Fig. 4B and Table II), presumably through activation of PMA-insensitive Ca\(^{2+}\)/H\(_{11001}\)-influx. Like T888Stop-mediated Ca\(^{2+}\)/H\(_{11001}\) responses, the PMA-insensitive IP responses of T888Stop have no significant difference from those of PMA-pretreated wild-type receptor (Fig. 3 and Table II).

To further test the hypothesis that CaR-induced elevation of Ca\(^{2+}\)/H\(_{11001}\) through activation of Ca\(^{2+}\)/H\(_{11001}\) influx markedly activates PLC, we stimulated the cells with spermine in the presence of 0.5 mM Ca\(^{2+}\)/H\(_{11001}\), a concentration below the threshold for activation of the CaR. The presence of 0.5 mM Ca\(^{2+}\)/H\(_{11001}\) increased the spermine-elicited maximal IP response of the wild-type receptor ~2.5-fold (Fig. 5A) to a level similar to that stimulated by high Ca\(^{2+}\)/H\(_{11001}\) (Fig. 4B). Moreover, the Ca\(^{2+}\)/H\(_{11001}\)-dependent IP productions were similar in cells transfected with T888Stop (Fig. 5B) to those in cells transfected with the wild-type receptor (Fig. 5A). These results further support that CaR-mediated Ca\(^{2+}\)/H\(_{11001}\) influx activates PLC and that the magnitude of CaR-elicited Ca\(^{2+}\)/H\(_{11001}\) influx in cells transfected with T888Stop is similar to that in cells transfected with the wild-type receptor. Importantly, these results also rule out the possibilities that spermine is significantly transported into the cells and subsequently inhibits intracellular PLC activities in HEK293 cells when applied extracellularly.

In contrast to T888Stop, T888V had maximal IP responses significantly higher than the wild-type receptor when stimulated with spermine or Ca\(^{2+}\)/H\(_{11001}\) (Fig. 6 and Table II). This result suggests that T888V primarily enhances the activity of the receptor for activation of pathways leading to mobilization of intracellular Ca\(^{2+}\) stores. Consistent with our studies on Ca\(^{2+}\)/H\(_{11001}\) responses, spermine- or Ca\(^{2+}\)/H\(_{11001}\)-elicited IP responses of T888V...
were also affected negatively by PMA. However, the IP response of T888V at any given concentration in the PMA-treated cells was not lower than that of the wild-type receptor in non-treated cells. This suggests that PMA exerts additional negative effect on signaling elements downstream of PLC activation that are involved in the release of intracellular Ca\textsuperscript{2+} stores.

Western analysis showed that all the mutant receptors had normal expression on the cell surface (Fig. 7). In fact, the truncated receptor had slightly higher expression than did the wild-type receptor.

**DISCUSSION**

Ca\textsuperscript{2+} is the main physiological regulator of calcium homeostasis through its action on its own cell surface receptor, i.e. the CaR. Increases in the concentration of Ca\textsuperscript{2+} elicit rapid transient Ca\textsuperscript{2+} responses followed by sustained increases in Ca\textsuperscript{2+} in parathyroid cells and CaR-transfected HEK293 cells. Previous studies have shown that both activation and inhibition of PKC have profound effects on Ca\textsuperscript{2+}-elicited Ca\textsuperscript{2+} responses in parathyroid cells (6, 7) and CaR-transfected HEK293 cells and that these effects can be significantly blocked by substituting Thr-888 with valine in the CaR (16).

In this study, we introduced a variety of amino acid substitutions for threonine at position 888 to test whether we can create a mutant receptor that resembles the PKC-phosphorylated receptor. We found that substitutions with hydrophobic amino acid residues such as alanine, phenylalanine, and tryptophan increased apparent receptor affinities to its agonists similar to those associated with valine substitution reported in our previous study (16). These increased apparent receptor affinities for its agonists, such as calcium and spermine, suggest that the CaR might be somewhat inhibited by basal PKC activity. In contrast, T888D, T888E, and T888G, the apparently less sensitive than the wild-type CaR to the receptor agonists with increased EC\textsubscript{50} values. The reason for T888E and T888D to simulate the wild-type receptor following PMA treatment is likely that the negatively charged amino acid residues may somewhat resemble the PKC-phosphorylated receptor with charges acquired at the phosphorylation site. In the case of T888G, substitution with glycine could significantly alter the secondary structure of the region that is important for the interaction of the receptor with its respective G proteins by introducing a turn in the helix, as predicted by Chou-Fasman indices. Regardless, all the mutant receptors with various point mutations elicited modest-to-high Ca\textsuperscript{2+} responses via mobilization of intracellular Ca\textsuperscript{2+} stores and showed significant but reduced sensitivities to PMA that may reflect the presence of additional PKC sites and/or more downstream modulation by PMA.

In contrast, the action of PKC phosphorylation can be fully mimicked by the removal of the C-terminal region following alanine at position 887, suggesting that PKC phosphorylation at Thr-888 blocks the interaction of this C-terminal region of the receptor with its cognate G proteins that leads to mobilization of intracellular Ca\textsuperscript{2+} stores. However, the removal of this C-terminal region of the receptor does not impede the receptor’s PMA-insensitive activation of Ca\textsuperscript{2+} influx and the subsequent Ca\textsuperscript{2+} dependent activation of PLC, suggesting that the structural determinants activating PMA-insensitive Ca\textsuperscript{2+} influx are intact in the truncated receptor, T888Stop. Since our previous study showed that truncation upstream of Thr-888 at position 877 completely inactivates not only CaR-mediated mobilization of intracellular Ca\textsuperscript{2+} stores but also CaR-mediated Ca\textsuperscript{2+} influx (24) the elements important for activation of Ca\textsuperscript{2+} influx are likely situated between the residues between 877 and 887.

For the wild-type CaR, activation of PMA-insensitive Ca\textsuperscript{2+} influx pathways becomes significant when the receptor is activated by a concentration of Ca\textsuperscript{2+} that is 3.5 mM or higher. The activation of this pathway increases not only the concentration of Ca\textsuperscript{2+}, but also the production of inositol phosphates. In fact, more than 85% of the IP response may be attributed to activation of Ca\textsuperscript{2+} influx when the receptor is stimulated by a concentration of Ca\textsuperscript{2+} that is 8 mM or higher. It becomes clear that at least 60% of the IP response is attributable to activation of Ca\textsuperscript{2+} influx when the receptor is activated by 1 or 2 mM spermine in the presence of 0.5 mM Ca\textsuperscript{2+}. Since all subtypes of PLC can be activated by Ca\textsuperscript{2+}, it is likely that increases in Ca\textsuperscript{2+}, due to activation of Ca\textsuperscript{2+} influx non-selectively activate all subtypes of PLC, including PLC-\beta that could be otherwise activated by G\textsubscript{i} or G\textsubscript{11}. Activating PLC via activation of PMA-insensitive Ca\textsuperscript{2+} influx pathways could play an important role in bone remodeling at the site of bone erosion, where the concentration of Ca\textsuperscript{2+} can be as high as 40 mM (23).

Interestingly, the IP responses of T888V were not significantly higher than those of the wild-type receptor but also much less sensitive to PMA than its own Ca\textsuperscript{2+}, responses. Moreover, the maximal IP response of T888V in PMA-treated cells was significantly higher than that of the wild-type receptor in non-treated cells. Hence, over 50% reduction in spermine-elicited Ca\textsuperscript{2+} responses of T888V may result from activation of PMA-sensitive Ca\textsuperscript{2+} stores, which can be inhibited by PMA. In contrast, T888Stop neither activates any PMA-sensitive Ca\textsuperscript{2+} stores nor fully activates PMA-insensitive stores. Therefore, PKC phosphorylation of the CaR inhibits both CaR-mediated activation of both PMA-sensitive and -insensitive Ca\textsuperscript{2+} stores.

In summary, we have demonstrated that PKC regulation of CaR-mediated PLC signaling pathways can be mimicked by truncating the cytoplasmic tail of the receptor at position 888. Therefore, PKC phosphorylation of the CaR impairs functional interaction of G proteins with the region of the cytoplasmic tail downstream of Ala-887.

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