We examined the role of protein kinase C (PKC) in the mechanism and regulation of intracellular Ca\(^{2+}\) oscillations elicited by an increase in the extracellular concentration of Ca\(^{2+}\) in human embryonic kidney 293 cells expressing the Ca\(^{2+}\)-sensing receptor (CaR). Exposure to the PKC inhibitors bisindolylmaleimide I (GF I) or Ro-31-8220 converted oscillatory responses to transient, non-oscillatory responses, significantly reducing the percentage of cells that showed [Ca\(^{2+}\)]\(_i\), oscillations but without decreasing the overall response to increase in [Ca\(^{2+}\)]\(_i\). Exposure to 100 nM phorbol 12,13-dibutyrate, a direct activator of PKC, eliminated [Ca\(^{2+}\)]\(_i\) oscillations. Addition of phorbol 12,13-dibutyrate at lower concentrations (3 and 10 nM) did not eliminate the oscillations but greatly reduced their frequency in a dose-dependent manner. Coexpression of CaR with constitutively active mutants of PKC (either \(\varepsilon\) or \(\beta_i\) isoforms) also reduced [Ca\(^{2+}\)]\(_i\) oscillation frequency. Expression of a mutant CaR in which the major PKC phosphorylation site is altered by substitution of alanine for threonine (T888A) eliminated oscillations mediated by this receptor.

The extracellular Ca\(^{2+}\)-sensing receptor (CaR) is a member of the superfamily of heptahelical G protein-coupled receptors (GPCRs) that was cloned originally from parathyroid chief cells (for review see Ref. 1). Inactivating and activating mutations of the CaR in humans (2) and genetic disruption of the CaR gene (for review see Ref. 1) have defined, physiological roles in the regulation of secretion, gene expression, cell proliferation, and apoptosis (1).

Most models proposed to explain the mechanism by which [Ca\(^{2+}\)]\(_i\) oscillations are generated in response to GPCR activation are based broadly on negative feedback effects of PKC on the production of Ins(1,4,5)P\(_3\) or on the regulatory properties of [Ca\(^{2+}\)]\(_i\) on the Ins(1,4,5)P\(_3\) receptor (17–19). For example, classic (\(\alpha, \beta_i, \beta_j, \) and \(\gamma\)) and/or novel (\(\delta, \epsilon, \eta, \) and \(\theta\)) isoforms of PKC, which are stimulated by [Ca\(^{2+}\)], and diacylglycerol or by diacylglycerol, respectively (20, 21), can attenuate phosphoinositide signaling either by phosphorylation and uncoupling of the receptor from Gq (22, 23) or by phosphorylation of the \(\beta_3\) isoform of phospholipase C, which prevents its activation by Gq (24). In addition, PKC can also reduce [Ca\(^{2+}\)]\(_i\), by accelerating the rate of Ca\(^{2+}\) extrusion from the cell (25). Our recent experiments, using the PKC inhibitor Ro-31-8220, suggested that negative feedback by PKC could also play a role in the generation of [Ca\(^{2+}\)]\(_i\),-evoked [Ca\(^{2+}\)]\(_i\) oscillations via the CaR (9). Specifically, in the presence of this inhibitor, most cells expressing CaR responded to an increase in [Ca\(^{2+}\)]\(_i\), by a transient increase in [Ca\(^{2+}\)], rather than by [Ca\(^{2+}\)]\(_i\), oscillations (9). In contrast, Breitwieser et al. (8) concluded that the activity of a variety of protein kinases, including PKC, does not influence the pattern of [Ca\(^{2+}\)], oscillations induced by activation of the CaR by [Ca\(^{2+}\)],. Thus, the mechanism(s) underlying [Ca\(^{2+}\)]\(_i\),-evoked [Ca\(^{2+}\)]\(_i\), oscillations through the CaR is not understood, and the role of protein kinases, especially of PKC, in this process remains controversial.

The studies presented here were designed to elucidate whether PKCs play a role in the generation of [Ca\(^{2+}\)]\(_i\), oscillations induced by activation of the CaR. Our results produced several lines of evidence indicating that PKCs negatively regulate the frequency of [Ca\(^{2+}\)]\(_i\), oscillations induced by activation of the CaR by increases in [Ca\(^{2+}\)]\(_i\). In particular, we demonstrate, for the first time, that expression of a mutant CaR in which the major PKC phosphorylation site (Thr-888) is altered...
by substitution of alanine for threonine completely eliminates [Ca\(^{2+}\)],-evoked [Ca\(^{2+}\)], oscillations. We conclude that [Ca\(^{2+}\)], oscillations induced by activation of the CaR in response to an increase in [Ca\(^{2+}\)], result from negative feedback involving PKC-mediated phosphorylation of the CaR at Thr-888.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK 293 cells were maintained in culture as described previously (26). For experimentation, cells were re-plated onto clean 15-mm diameter number 1 glass coverslips (Warner Instrument Corp., Hamden, CT) resting on the bottom of 35-mm plastic culture dishes 1 day before transfection. Approximately 8.5 \times 10^4 cells were seeded per 35-mm dish. Culture medium was high glucose Dulbecco’s modified Eagle’s medium (D5796; Sigma) supplemented with 10% fetal bovine serum, penicillin (10 units/ml), streptomycin (10 \mu g/ml), and amphotericin B (25 ng/ml). Cells were maintained in a humidified incubator at 37 \degree C.

**Transient Transfection**—A total of 1.7 \mu g of plasmid DNA (human CaR cDNA cloned in the pCR3.1 expression vector) was kindly provided by Dr. Allen Spiegel, NIDDK, National Institutes of Health, Bethesda, MD) was used to transfect cells in each 35-mm culture dish. DNA was diluted and mixed with Lipofectin (Invitrogen) according to manufacturer’s guidelines. The resulting solution (volume, 1 ml) was added to the cells, and after 4–6 h, cells were supplemented with 10% fetal bovine serum. Cells were studied 2 or 3 days after transfection. To identify transfected cells expressing the CaR, co-transfections were performed with vector encoding CaR and expression vector pDsRed1-N1 (Invitrogen), which produces a red fluorescent protein. In all co-transfections, total DNA was maintained at 1.7 \mu g.

**Construction of CaR Mutant—CaR\(^{T888A}\) mutant was constructed by QuikChange™ mutagenesis (Strategene) using cloned human CaR cDNA plasmid as a template and a pair of complementary primers (sense, 5’-CAAGGTTGCTGCGCGCCGCCGCTGCAGCCGAAG; antisense, 5’TGCCTGCGCGCCGCCAGCCGGAGCAAGCCTTG) in which the codon for glutamic acid at 888 was replaced with alanine.

**Solutions**—Physiological saline consisted of Hank’s balanced salt solution (HBSS; Invitrogen) without phenol red supplemented with 25 mM HEPES buffer. Final concentrations (in mM) were as follows: 138 NaCl, 4 NaHCO\(_3\), 0.3 Na\(_2\)HPO\(_4\), 5 KCl, 0.3 KH\(_2\)PO\(_4\), 1.5 CaCl\(_2\), 0.5 MgCl\(_2\), 0.4 MgSO\(_4\), 5.6 D-glucose, 20 HEPES, pH 7.4.

**Measurement of [Ca\(^{2+}\)]**—Cells on coverslips were loaded with the fluorescent calcium indicator Fura-2 by incubation in saline containing 5 \mu M Fura-2/AM (Molecular Probes, Eugene, OR) for 45–60 min at 37 \degree C. Coverslips were then mounted in an experimental chamber (RC-25F; Warner Instrument Corp.), which was perfused at 1.5 ml/min. The perfusion solution was heated using an inline heater (TC-34B; Warner Instrument Corp.), which maintained bath temperature at 37 \degree C. The chamber was in turn placed on the stage of an inverted microscope (Zeiss TV 100; Carl Zeiss, Inc., Thornwood, NY) to which was attached a digital imaging system (Axiovert; Atto Instruments, Rockville, MD) with electronically controlled excitation filter positions and associated software (RatioVision). Ratios of images (340 nm excitation/380 nm emission, filter 520 nm) were obtained at 1.5 s intervals. A region of interest covering 15 \times 15 \mu m was defined over each cell, and the average ratio intensity over the region was converted to [Ca\(^{2+}\)], using a calibration curve constructed with a series of calibrated buffered calcium solutions (calcium calibration buffer kit #2; Molecular Probes). For each cell, [Ca\(^{2+}\)], values and their times of acquisition were stored on computer disk. All experiments were performed at 37 \degree C.

**Materials**—GF I (bisindolylmaleimide 1), GF V (bisindolylmaleimide V), and PD98059 (phorbol-12,13-dibutyrate) were purchased from Calbiochem. Plasmids encoding constitutively active mutant PKC isoforms ε\(_{\text{mut}}\) and β\(_{\text{mut}}\) were kindly provided by Dr. Peter Parker, Imperial Cancer Research Institute, London, United Kingdom.

**RESULTS**

**The PKC Inhibitors Ro-31-8220 and GF I Reduce [Ca\(^{2+}\)], Oscillations without Reducing Overall [Ca\(^{2+}\)], Response**—To examine the effect of [Ca\(^{2+}\)], on CaR-mediated changes in [Ca\(^{2+}\)], in single cells, HEK 293 cells transiently transfected with the human CaR cDNA were loaded with the fluorescent Ca\(^{2+}\) indicator Fura-2 and incubated in the presence of 1.5 mM [Ca\(^{2+}\)]. Intracellular Ca\(^{2+}\) imaging revealed that most transfected cells exhibited a stable [Ca\(^{2+}\)]. In agreement with our recent results (9), a small increase in [Ca\(^{2+}\)], from 1.5 to 3.0 mM elicited sustained [Ca\(^{2+}\)], oscillations (Fig. 1a). The fraction of responsive cells was 72%, and that fraction displaying [Ca\(^{2+}\)], oscillations was 57% (n = 28; see Fig. 1d). Untransfected HEK 293 cells or cells transfected with vector did not exhibit [Ca\(^{2+}\)], oscillations in response to an identical increase in [Ca\(^{2+}\)].
Ca\(^{2+}\) Oscillations by the CaR Need Negative Feedback by PKC

To determine whether PKC activity is required for the generation of [Ca\(^{2+}\)]\(_{o}\) oscillations, HEK 293 cells transfected with CaR were pretreated for 1 h with the selective PKC inhibitors Ro-31-8220 (27) or GF I (28, 29). An increase in [Ca\(^{2+}\)]\(_{o}\) in cells exposed to Ro-31-8220 at 1.25 \(\mu\)M induced a marked increase in [Ca\(^{2+}\)]\(_{i}\), but the change in [Ca\(^{2+}\)]\(_{o}\) no longer had a strong oscillatory component; the majority of the responses consisted of an initial [Ca\(^{2+}\)]\(_{i}\), transient followed by a sustained plateau. Individual traces from four separate cells are presented in Fig. 1b. Pretreatment with GF I (3.5 \(\mu\)M) also inhibited the oscillatory behavior induced by an increase in [Ca\(^{2+}\)]\(_{o}\), and also resulted in a sustained increase in [Ca\(^{2+}\)]\(_{i}\), after the increase in [Ca\(^{2+}\)]\(_{o}\). Individual traces from four separate cells are illustrated in Fig. 1c. As summarized in Fig. 1d, the fraction of GF I-treated cells that exhibited [Ca\(^{2+}\)]\(_{o}\) oscillations dropped to only 16% (\(p < 0.05\) compared with control; \(n = 14\)) whereas the percentage of responsive cells treated with GF I was 78%. Thus, the PKC inhibitor blunted the oscillatory behavior without reducing overall responsiveness. In contrast, exposure of HEK 293 cells transfected with CaR to 3.5 \(\mu\)M GF V (an inactive analog of GF I) for 1 h did not have any significant inhibitory effect on the fraction of cells that displayed [Ca\(^{2+}\)]\(_{i}\)-evoked [Ca\(^{2+}\)]\(_{o}\) oscillations (\(p > 0.05\); \(n = 35\)).

A previous study (8) has reported that treatment with GF I did not produce any effect on the pattern of [Ca\(^{2+}\)]\(_{o}\) oscillations mediated by the CaR in HEK 293 cells. In the study of Brettwieser et al. (8), the cells were treated with 1 \(\mu\)M GF I for 4 min at 22 °C. When we used GF I at identical experimental conditions (i.e. 1 \(\mu\)M at 22 °C for 4 min), we also observed that such an exposure to this agent did not inhibit [Ca\(^{2+}\)]\(_{i}\) oscillations in response to an increase in [Ca\(^{2+}\)]\(_{o}\) in HEK 293 cells expressing the CaR. In this case we found that the fraction of cells showing [Ca\(^{2+}\)]\(_{i}\) oscillations was 53%, and the percentage of responsive cells was 69%, similar to control values (Fig. 1d). It is likely that under these conditions exposure to GF I did not change [Ca\(^{2+}\)]\(_{i}\) oscillations, because it did not accumulate in the cell at a sufficient concentration.

The Frequency of [Ca\(^{2+}\)]\(_{o}\), Oscillations Induced by [Ca\(^{2+}\)]\(_{o}\). Stimulation of the CaR is Modulated by PDBu, an Activator of PKC—Previous studies using cell populations showed that phorbol ester-induced activation of PKC abrogates [Ca\(^{2+}\)]\(_{i}\)-evoked stimulation of phospholipase C and the associated increases in [Ca\(^{2+}\)]\(_{i}\), suggesting that PKC may modulate the coupling of the CaR to intracellular signaling systems directly (30). Accordingly, we found that addition of 100 nM PDB to HEK 293 cells expressing CaR stops [Ca\(^{2+}\)]\(_{i}\)-induced [Ca\(^{2+}\)]\(_{o}\) oscillations within a few cycles (Fig. 2a).

If negative feedback by PKC is required for the production of [Ca\(^{2+}\)]\(_{i}\)-induced [Ca\(^{2+}\)]\(_{o}\) oscillations, we would expect that exposure to lower concentrations of PDBu (e.g. 3 and 10 nM) should modulate oscillation frequency. As shown in Fig. 2b, pretreatment of the cells with 3 nM PDBu for 5 min reduced average oscillation frequency. Increasing the concentration of PDBu to 10 nM further reduced [Ca\(^{2+}\)]\(_{o}\) oscillation frequency in response to an elevation of [Ca\(^{2+}\)]\(_{o}\) (Fig. 2c). The results are summarized in Fig. 2d. The average [Ca\(^{2+}\)]\(_{i}\) oscillation frequency dropped by about 50% after 5 min of exposure to 3 nM PDBu (control, 2.7 ± 0.14/min (± S.E.), \(n = 28\); 3 nM PDBu, 1.39 ± 0.16/min (± S.E.), \(n = 21\); \(p < 0.0001\)). An increase in PDBu concentration to 10 nM caused a further reduction in oscillation frequency (0.74 ± 1.6/min (± S.E.), \(n = 12\); \(p < 0.02\) compared with 3 nM PDBu). These results suggest that PDBu-induced PKC activation reinforces a negative feedback component that markedly reduces the frequency of [Ca\(^{2+}\)]\(_{i}\)-induced [Ca\(^{2+}\)]\(_{o}\) oscillations mediated by the CaR in single cells.

The Frequency of [Ca\(^{2+}\)]\(_{o}\), Oscillations Induced by [Ca\(^{2+}\)]\(_{o}\). Stimulation of the CaR Is Modulated by Expression of Constitutively Active Mutant Isoforms of PKC—Having established that pharmacological inhibition or activation of PKC's strikingly influences the frequency of [Ca\(^{2+}\)]\(_{i}\)-induced [Ca\(^{2+}\)]\(_{o}\) oscillations in HEK 293 cells expressing the CaR, our next step was to examine whether overexpression of PKC isoforms also down-regulates the frequency of [Ca\(^{2+}\)]\(_{i}\)-induced [Ca\(^{2+}\)]\(_{o}\) oscillations mediated by the CaR. The PKCs comprise a family of at least 10 distinct isoforms, which can be classified into three distinct subgroups on the basis of structural and regulatory differences (31–33). Classic PKCs (\(\alpha, \beta, \) and \(\gamma\)) respond to intracellular
**Ca$$^{2+}$$ Oscillations by the CaR Need Negative Feedback by PKC**

![Graph showing Ca$$^{2+}$$ oscillations](image)

**Fig. 3. Co-transfection of HEK cells with plasmids coding for constitutively active (mutant) PKC isoforms $\epsilon$ or $\beta_1$ reduces oscillation frequency.** a, control cell. HEK 293 cells were transfected with a plasmid encoding the CaR, as described under “Experimental Procedures.” The increase of [Ca$$^{2+}$$] to 3.0 mM from a resting concentration of 1.5 mM is marked by a horizontal line below the [Ca$$^{2+}$$] trace. The increase in [Ca$$^{2+}$$] triggered striking oscillations in [Ca$$^{2+}$$]. b, cell transfected with active $\beta_1$ form (PKC$$^{\beta_1\text{mut}}$$) showed reduced [Ca$$^{2+}$$], oscillation frequency after [Ca$$^{2+}$$] was raised from 1.5 to 3.0 mM. c, summary of oscillation frequencies. Cells that were co-transfected with plasmids encoding for either active PKC $\epsilon$ (PKC$$^{\epsilon\text{mut}}$$) or active PKC $\beta_1$ form (PKC$$^{\beta_1\text{mut}}$$) exhibited a decrease in average [Ca$$^{2+}$$], oscillation frequency. Co-transfection of cells with plasmid coding for protein kinase D (PKD) produced [Ca$$^{2+}$$], oscillations whose frequency did not differ from cells that were transfected with empty vector or from untransfected cells (control), $p$ values are compared with control, and error bars represent S.E.

Ca$$^{2+}$$ rises in diacylglycerol and [Ca$$^{2+}$$], whereas novel PKCs ($\delta$, $\epsilon$, $\eta$, and $\theta$) respond to diacylglycerol but not to changes in [Ca$$^{2+}$$]. Receptor stimuli that induce [Ca$$^{2+}$$], oscillations promote a parallel repetitive translocation of classic PKCs to the plasma membrane in a variety of model systems (11, 23, 34). In contrast, novel PKCs translocate to the plasma membrane with kinetics that are not synchronized with [Ca$$^{2+}$$], oscillations (35). These considerations prompted us to examine the effect of co-transfection of the CaR with either a classic or a novel isoform of the PK family.

Cultures of HEK 293 cells were co-transfected with plasmids encoding the CaR and either vector, constitutively active isoforms of PKC ($\beta_1$ and $\epsilon$), or protein kinase D, a kinase that is activated downstream of PKC (36–39). Fig. 3a shows a typical tracing of [Ca$$^{2+}$$], oscillations in a control HEK 293 cell transfected with the CaR after [Ca$$^{2+}$$], was raised from 1.5 to 3.0 mM. As shown in Fig. 3b, when HEK 293 cells were co-transfected with plasmids encoding for the CaR and constitutively active PKC isoform $\beta_1$ (PKC$$^{\beta_1\text{mut}}$$), the oscillation frequency was reduced markedly. The results are summarized in Fig. 3c. Average oscillation frequencies of cells transfected with an empty vector or cells transfected with a plasmid encoding protein kinase D were not significantly different ($p > 0.5$, $n = 17$ cells; $p > 0.3$, $n = 30$ cells) from control cells transfected with only the plasmid encoding CaR. In contrast, cells transfected with the active mutants of PKC isoforms $\epsilon$ (PKC$$^{\epsilon\text{mut}}$$) or $\beta_1$ (PKC$$^{\beta_1\text{mut}}$$) show reduced average oscillation frequencies compared with control cells ($p, p = 0.001, n = 32$ cells). These results suggest that an increase in the cellular concentration of PKC reinforces a negative feedback loop that regulates the frequency of [Ca$$^{2+}$$]$$^\text{i}$-induced [Ca$$^{2+}$$]$$^\text{i}$$ oscillations in HEK 293 cells expressing the CaR.

Mutation of Threonine 888, the Predominant PKC Phosphorylation Site of the CaR, Prevents [Ca$$^{2+}$$]$$^\text{i}$$ Oscillations—In a previous study using cell populations, Bai et al. (30) have shown that PKC-mediated phosphorylation of threonine 888 of the CaR inhibits [Ca$$^{2+}$$]$$^\text{i}$$-induced increase in [Ca$$^{2+}$$]$$^\text{i}$, indicating that this amino acid is the major site for the inhibitory influence of PKC on the CaR. However, the effect of this mutation on the oscillatory signaling properties of the CaR in individual living cells has not been investigated.

If CaR-mediated [Ca$$^{2+}$$]$$^\text{i}$$ oscillations are generated physiologically by the periodic phosphorylation of this inhibitory site by PKC, mutation of Thr-888 to a non-phosphorylatable amino acid should significantly reduce or even eliminate [Ca$$^{2+}$$]$$^\text{i}$$ oscillations mediated by this receptor. To test this hypothesis, we expressed a CaR in which the threonine at position 888 of the CaR was mutated to alanine (T888A). As shown in Fig. 4, HEK 293 cells expressing the CaR$$^{\text{T888A}}$$ did not show [Ca$$^{2+}$$]$$^\text{i}$$ oscillations after CaR activation by increases in [Ca$$^{2+}$$]$$^\text{i}$, followed by a sustained phase of elevated [Ca$$^{2+}$$]$$^\text{i}$. In all, of 129 responding cells analyzed from five independent preparations that responded to an increase in [Ca$$^{2+}$$]$$^\text{i}$$ to 3.0 mM, all 129 showed this behavior, i.e. none exhibited [Ca$$^{2+}$$]$$^\text{i}$$ oscillations (Fig. 4c).

In addition, prior exposure to PDBu (100 nM for 5 min) does not prevent the response to 3.0 mM [Ca$$^{2+}$$]$$^\text{i}$, in CaR$$^{\text{T888A}}$$-expressing cells (Fig. 4d). We verified that the non-oscillatory [Ca$$^{2+}$$]$$^\text{i}$, response induced by an increase in [Ca$$^{2+}$$]$$^\text{i}$$ to 3.0 mM in cells expressing this CaR$$^{\text{T888A}}$ mutant in HEK 293 cells did not prevent the [Ca$$^{2+}$$]$$^\text{i}$$, oscillations induced by 10 $\mu$M carbachol (data not shown), which acts via an endogenous muscarinic GPCR expressed by these cells (40).

We have reported recently that stimulation of HEK 293 cells expressing the CaR with amino acids (l-phenylalanine or l-tryptophan) induced [Ca$$^{2+}$$]$$^\text{i}$$ oscillations of a different pattern from [Ca$$^{2+}$$]$$^\text{i}$$, oscillations induced by increases in [Ca$$^{2+}$$]$$^\text{i}$$ (9). The patterns differed in the following ways: 1) The amino acid-induced [Ca$$^{2+}$$]$$^\text{i}$$ transients repetitively returned to baseline levels. 2) The amino acid-induced oscillation frequencies (1/min) were lower than those induced by [Ca$$^{2+}$$]$$^\text{i}$$ (4/min).

Amino acid-induced and [Ca$$^{2+}$$]$$^\text{i}$$-induced oscillations differed in their sensitivity to pharmacological agents that influence [Ca$$^{2+}$$]$$^\text{i}$, (9). The results illustrated in Fig. 5a confirm that stimulation of HEK 293 cells expressing the wild type CaR with 5 mM l-phenylalanine induced [Ca$$^{2+}$$]$$^\text{i}$$ oscillations of a different pattern from [Ca$$^{2+}$$]$$^\text{i}$$, oscillations induced by an increase in [Ca$$^{2+}$$]$$^\text{i}$$, (from 1.5 to 3 mM). These results raised the interesting
Oscillations by the CaR Need Negative Feedback by PKC

the CaR through a different mechanism(s). In view of these considerations and the results illustrated in Fig. 4 with CaR<sup>T888A</sup>, we examined [Ca<sup>2+</sup>]<sup>i</sup>, oscillations triggered by addition of 5 mM L-phenylalanine to cultures of HEK 293 cells expressing either wild type CaR or CaR<sup>T888A</sup>. As shown in Fig. 5b, [Ca<sup>2+</sup>]<sup>i</sup>, oscillations induced by addition of 5 mM L-phenylalanine persisted in the same cells expressing CaR<sup>T888A</sup> in which [Ca<sup>2+</sup>]<sup>i</sup>,-induced [Ca<sup>2+</sup>]<sup>i</sup>, oscillations were eliminated, although the average oscillation frequency of the l-phenylalanine-induced [Ca<sup>2+</sup>]<sup>i</sup>, oscillations was reduced (1.0 ± 0.06 min<sup>-1</sup>; n = 23 cells) compared with wild-type CaR (1.3 ± 0.1 min<sup>-1</sup>; n = 14 cells). These results provide further support for the notion that amino acids and [Ca<sup>2+</sup>]<sup>i</sup>, produce [Ca<sup>2+</sup>]<sup>i</sup>, oscillations through the CaR via different mechanisms.

**DISCUSSION**

Most models proposed to explain the mechanism by which [Ca<sup>2+</sup>]<sup>i</sup>, oscillations are generated in response to GPCR activation are based broadly on negative feedback effects of PKC on the production of Ins(1,4,5)P<sub>3</sub> or on the regulatory properties of [Ca<sup>2+</sup>]<sup>i</sup>, on the Ins(1,4,5)P<sub>3</sub> receptor (17–19). However, definitive evidence identifying the mechanism(s) involved is available in very few instances. For example, even the role of PKC in the generation of glutamate-induced [Ca<sup>2+</sup>]<sup>i</sup>, oscillations mediated by the metabotropic glutamate receptor 5, one of the most studied systems, appears controversial (22, 23, 34). In the present study, we examined the hypothesis that PKC activation provides a negative feedback link that is critical for generating the [Ca<sup>2+</sup>]<sup>i</sup>, oscillatory behavior produced by [Ca<sup>2+</sup>]<sup>i</sup>, activation of the CaR.

Our results produced several lines of evidence indicating that PKCs negatively regulate the frequency of [Ca<sup>2+</sup>]<sup>i</sup>, oscillations induced by activation of the CaR by increases in [Ca<sup>2+</sup>]<sup>i</sup>,. 1) Exposure to the PKC inhibitors GF I or Ro-31-8220 converted oscillatory responses to transient, non-oscillatory responses, significantly reducing the percentage of cells that
showed [Ca\textsuperscript{2+}], oscillations but without decreasing the overall response to [Ca\textsuperscript{2+}]. 2) Exposure to PDBu, a direct activator of PKC, greatly reduced the frequency of [Ca\textsuperscript{2+}], oscillations in a dose-dependent manner. 3) Co-expression of CaR with constitutively active mutants of PKC (either e or b) isoforms reduced [Ca\textsuperscript{2+}], oscillation frequency. These results indicate that [Ca\textsuperscript{2+}], oscillations generated in response to [Ca\textsuperscript{2+}]\textsubscript{i}-elicited CaR activation are based on negative feedback effects of PKC.

PKC can exert negative feedback on phosphoinositide signaling either by phosphorylation and uncoupling of the receptor from Gq (22, 23) or by phosphorylation of the b\textsubscript{3} isofrom of phospholipase C, which prevents its activation by Gq (24). In addition, PKC can also reduce [Ca\textsuperscript{2+}], by accelerating the rate of Ca\textsuperscript{2+}\textsubscript{i} extrusion from the cell (25). A salient feature of the results presented here is that a single amino acid substitution in the CaR was sufficient to drastically reduce the ability of this GPCR to generate [Ca\textsuperscript{2+}], oscillations. Specifically, mutation of the major PKC phosphorylation site, threonine 888, to alanine (26) eliminates [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. This suggests that the pattern and frequency of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations induced by L-phenylalanine and [Ca\textsuperscript{2+}]\textsubscript{i} in response to receptor stimulation are not controlled by PKC providing negative feedback through CaR activation. It is plausible that the pattern of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations triggered by activation of the CaR in response to an increase in [Ca\textsuperscript{2+}], could be finely tuned by PKC activity, which in turn is determined by other signals reaching the cell.

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