The agonist sensitivity of the calcium-sensing receptor (CaR) can be altered by protein kinase C (PKC), with CaR residue Thr^{888} contributing significantly to this effect. To determine whether CaR^{Thr888} is a substrate for PKC and whether receptor activation modulates such phosphorylation, a phospho-specific antibody against this residue was raised (CaRp^{Thr888}). In HEK-293 cells stably expressing CaR (CaR-HEK), but not in cells expressing the mutant receptor CaR^{Thr888A}, phorbol ester (PMA) treatment increased CaR^{Thr888} immunoreactivity as observed by immunoblotting and immunofluorescence. Raising extracellular Ca^{2+} concentration from 0.5 to 2.5 mM increased CaR^{Thr888} phosphorylation, an effect that was potentiated stereospecifically by the calcimimetic NPS R-467. These responses were mimicked by 5 mM extracellular Ca^{2+} and abolished by the calcilytic NPS-89636 and also by PKC inhibition or chronic PMA pretreatment. Whereas CaR^{Thr888A} did exhibit apparent agonist sensitivity, by converting intracellular Ca^{2+} to CaR^{Thr888}, oscillations to sustained plateau responses in some cells, we still observed Ca^{2+} oscillations in a significant number of cells. This suggests that CaR^{Thr888} contributes significantly to CaR regulation but is not the exclusive determinant of CaR-induced Ca^{2+} oscillations. Finally, dephosphorylation of CaR^{Thr888} was blocked by the protein phosphatase 1/2A inhibitor calyculin, a treatment that also inhibited Ca^{2+} oscillations. In bovine parathyroid cells, therefore, CaR^{Thr888} is a substrate for receptor-induced, PKC-mediated feedback phosphorylation and can be dephosphorylated by a calyculin-sensitive phosphatase.

The extracellular calcium-sensing receptor (CaR)^2 is a type III G protein-coupled receptor, whose primary function is to regulate parathyroid hormone (PTH) secretion and thus whole body calcium homeostasis (1, 2). The parathyroid CaR acts by responding to elevated extracellular Ca^{2+} (Ca^{2+}_i) in its intracellular domain (Thr^{888}, Ser^{895}, and Ser^{915}) (6). Substitution of Thr^{888} with negatively charged amino acids mimics the effect of phorbol ester treatment on wild-type CaR (8). There is also evidence that CaR^{Thr888A} elicits sustained Ca^{2+}_i mobilization rather than oscillations (9).

Whereas this suggests that PKC-mediated CaR phosphorylation regulates CaR function, no reagents have existed previously for investigating CaR phosphorylation directly. Thus, we have developed a phospho-specific antibody that recognizes the phosphorylated form of CaR^{Thr888}, this residue being chosen because its mutation elicited the greatest effect on CaR function. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2. The abbreviations used are: CaR, calcium-sensing receptor; PTH, parathyroid hormone; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2, protein phosphatase 2.
bovine serum (Invitrogen Ltd., Paisley, Scotland, UK) and 200 μg/ml hygromycin B (Roche Applied Science).

**Phosphorylation Assay**—Cells were grown to 80–90% confluence in 35-mm culture dishes and assayed as described previously (10, 11). Briefly, cells were rinsed in phosphate-buffered saline for 5 min and solubilized for 20 min in Experimental Buffer (20 mM HEPES (pH 7.4), 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, and 5.5 mM glucose) at 37 °C. Cells were then exposed to various experimental treatments for 10 min and then lysed on ice in RIPA buffer (12 mM HEPES (pH 7.6), 300 mM mannitol, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1.25 μM pepstatin, 4 μM leupeptin, 4.8 μM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 100 μM vanadate, 1 mM NaF, and 250 μM sodium pyrophosphate). In all experiments where CaCl₂ concentration was increased, the concentration of NaCl was reduced accordingly to normalize osmolality. Finally, cell lysate was then mixed with 5× Laemmli buffer and heated at 65 °C for 3 min prior to immunoblotting.

**Immunoblotting and Immunocytochemistry**—Immunoblotting was performed as described previously (10, 11). Anti-CaR mouse monoclonal antibody, raised to amino acids 214–235 (ADD) of the extracellular domain of the human parathyroid CaR was from Affinity Bioreagents (Golden, CO) and anti-protein phosphatase 2A (catalytic subunit) monoclonal antibody was from Upstate (Millipore, Chandlers Ford, Hampshire, UK). Phosphorylation of CaR residue Thr⁸⁸⁸ was studied using a custom-generated (Sigma Genosys) polyclonal antibody raised to the phosphorylated form of Thr⁸⁸⁸ contained within amino acids 882–896 of the human CaR sequence (KVAARA(pT)LRRSNVSR). The antibody was affinity purified using the non-phosphorylated peptide to remove non-phosphospecific antibody, followed by collection of the phosphospecific antibody (1.1 mg/ml) on a column prepared using the phosphorylated peptide. For immunoprecipitation experiments, cell lysates were mixed with protein A-Sepharose (1:100) followed by Protein A-Sepharose overnight (4 °C with constant rotation). The immunoprecipitates were collected by centrifugation and washed 3 times in SDS-free RIPA buffer prior to immunoblotting as before.

For immunofluorescence, cells were grown on coverslips to 50–80% confluence and fixed with 10% (w/v) paraformaldehyde solution at room temperature for 30 min and permeabilized with 0.075% (w/v) saponin in phosphate-buffered saline for 10 min. Indirect immunofluorescence was performed using the anti-CaR⁸⁸⁸ polyclonal antibody (1:200 dilution) and an Alexa 488-conjugated goat anti-rabbit secondary antibody (1:1000; Molecular Probes). Alternatively, anti-CaR monoclonal (ADD; 1:200) was used with a donkey anti-mouse secondary antibody conjugated with Alexa 594 (1:1000; Molecular Probes). In some experiments the primary antibody was preincubated overnight at 4 °C with an excess of antigenic peptide (either phosphorylated or non-phosphorylated) prior to incubation with the cells. Immunofluorescence was examined using a Zeiss Axioplan 2 fluorescence microscope with images acquired using a Hamamatsu digital camera, with each sample imaged under identical exposure conditions. Images were processed using the software package KS300 version 3.0 (Carl Zeiss Ltd., Hertfordshire, UK).

**Intracellular Ca²⁺ Assay**—CaR-HEK cells were cultured on glass coverslips and loaded with Fura-2/AM (1 μM for 1 h) at room temperature in the dark in Ca²⁺ assay buffer (20 mM HEPES, pH 7.4, 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 5.5 mM glucose) supplemented with 0.1% bovine serum albumin. Non-absorbed Fura-2/AM was removed by washing and the cells were equilibrated for 10 min in Experimental Buffer containing the baseline [Ca²⁺]₀, appropriate for the ensuing experiment. The cells were mounted in a perfusion chamber (Warner Instruments, Hamden, CT) and observed through a ×40 oil-immersion objective. Dual-excitation wavelength microfluorometry was then performed using a Nikon Diaphot inverted microscope (Cairn Research Ltd., Kent, UK). Experiments were performed at room temperature in Ca²⁺ assay buffer containing various concentrations of CaCl₂ (0.5 mM unless otherwise stated).

**Statistical Analysis**—Data are presented as mean ± S.E. and statistical significance was determined by one-way analysis of variance (Tukey post hoc test) or by unpaired t test as appropriate.
CaR<sup>T888</sup> Phosphorylation

**RESULTS**

CaR-HEK cells were treated with the phorbol ester, phorbol 12-myristate 13-acetate (PMA) to stimulate PKC activity and the resulting change in CaR<sup>T888</sup> phosphorylation was investigated by immunoblotting and immunofluorescence. The affinity purified, phospho-specific anti-CaR<sup>T888</sup> antibody detected a PMA-induced increase in immunoreactivity in two protein bands (Fig. 1A, panel i) corresponding to the high-mannose “core”-glycosylated CaR (140 kDa) and the mature glycosylated CaR (160 kDa) (12). To confirm the specificity of the signal, the anti-CaR<sup>T888</sup> antibody was used to stain CaR-HEK cells and the resulting change in CaR<sup>T888</sup> phosphorylation was investigated using either CaR<sup>T888</sup>-specific antibody (A and B, upper panels) or total CaR antibody (B, lower panels) as described in the legend to Fig. 1. Results shown are representative of a minimum of three independent experiments.

**FIGURE 2.** CaR<sup>T888</sup> immunoreactivity in cells expressing wild-type or mutant CaR. HEK cells stably transfected with either wild-type CaR (wt) or CaR<sup>T888A</sup> mutant (T888A) receptors were PMA-treated and processed either for immunoblotting (A) or immunofluorescence (B) using either CaR<sup>T888</sup>-specific antibody (A and B, upper panels) or total CaR antibody (B, lower panels) as described in the legend to Fig. 1. Results shown are representative of a minimum of three independent experiments.

**FIGURE 3.** Agonist-induced CaR<sup>T888</sup> phosphorylation. A, CaR-HEK cells were preincubated in the presence or absence of 250 nM GF109203X (5 min), then exposed to Experimental Buffer supplemented with either an additional 2 mM Ca<sup>2+</sup> (2.5 mM final), 1 μM NPS R-467, GF109203X, or a combination of treatments as indicated, for further 10 min. Immunoblotting was then performed on the resulting cell lysates using anti-CaR<sup>T888</sup> antibody and quantified by densitometry. Data are shown histographically (n = 4) below a representative blot. *, p < 0.05 versus control; ++, p < 0.001 versus 2.5 Ca<sup>2+</sup>/NPS R-467. B, cells treated as in A were processed for immunofluorescence. Results are representative of a minimum of three independent experiments.

**FIGURE 3.**

CaR<sup>T888</sup> phosphorylation is altered by receptor stimulation, CaR-HEK cells were incubated for 10 min in buffer containing increasing Ca<sup>2+</sup> concentrations in the presence or absence of the calcimimetic NPS R-467 (CaR positive allosteric modulator). Increasing Ca<sup>2+</sup> concentration from 0.5 to 2.5 mM induced a small rise in CaR<sup>T888</sup> phosphorylation (Fig. 3A), whereas addition of NPS 467-R (1 μM) increased it further (Fig. 3, A and B; 42 ± 10%, p < 0.05). Cotreatment with the PKC inhibitor GF109203X inhibited the signal substantially (−60 ± 3%, p < 0.001). These responses were qualitatively similar to the immunofluorescence observed in situ in CaR-HEK cells grown on cover-
slips and stained as before. Under control conditions (0.5 mM $\text{Ca}^{2+}$-containing buffer), NPS 467 and GF109203X were without effect (data not shown). None of the above responses were observed in HEK cells stably transfected with empty vector (data not shown).

The effect of NPS R-467 on CaR$^{T888}$ phosphorylation was stereoselective as 1 $\mu$m NPS S-467 was without effect (Fig. 4). Replacement of NPS R-467 with an additional 2.5 mM $\text{CaCl}_2$ (i.e. 5 mM final $\text{Ca}^{2+}$ concentration) also elicited additional CaR$^{T888}$ phosphorylation and this effect was abolished by cotreatment with the calcilytic NPS-89636 (1 $\mu$m; CaR negative allosteric modulator). High $\text{Ca}^{2+}$-induced CaR$^{T888}$ phosphorylation was also observed in cells treated at room temperature (data not shown).

Previous studies have shown that the mutant receptor CaR$^{T888A}$ exhibits greater sensitivity to $\text{Ca}^{2+}$ than the wild-type receptor (8) and that stimulation of CaR$^{T888A}$ with 3 mM $\text{Ca}^{2+}$ elicits non-oscillatory, sustained $\text{Ca}^{2+}$ mobilization compared with the oscillatory behavior observed with the wild-type receptor (9). To examine this further, we compared the responses of wild-type CaR and CaR$^{T888A}$ to various concentrations of $\text{Ca}^{2+}$, using single-cell epifluorescence $\text{Ca}^{2+}$ imaging (Fig. 5). In wild-type CaR-HEK cells, increasing $\text{Ca}^{2+}$ concentration from 0.5 to 2 mM induced a transient response in some cells (16%), whereas most of the others were non-responsive (Fig. 5, A, panel i, and B). However, in cells stably transfected with CaR$^{T888A}$, 2 mM $\text{Ca}^{2+}$ elicited responses in 58% of cells including slow oscillations and sustained responses (Fig. 5, A, panel ii, and B). Following subsequent treatment with 3 mM $\text{Ca}^{2+}$, virtually all of the CaR-HEK cells responded, with the majority exhibiting oscillations although with 32% showing sustained $\text{Ca}^{2+}$ mobilization. In the CaR$^{T888A}$-HEK cells, the majority of responsive cells exhibited sustained responses although 25% still exhibited slow oscillations. To compare the $\text{Ca}^{2+}$ sensitivity of CaR$^{T888A}$ versus wild-type receptor, the dose-response data for each cell were pooled, revealing that the $EC_{50}$ of 3.4 mM for wild-type CaR was reduced to 2.6 mM $\text{Ca}^{2+}$ in the CaR$^{T888A}$ cells, consistent with a previous study in which the receptors were expressed transiently (8). CaR-induced $\text{Ca}^{2+}$ oscillations initiated by 100 $\mu$m neomycin were maintained for up to 5 min in both $\text{Ca}^{2+}$-containing (0.5 mM) and $\text{Ca}^{2+}$-free (supplemented with 1 mM EGTA) buffer but were abolished under $\text{Ca}^{2+}$-free conditions by thapsigargin pretreatment demonstrating the involvement of intracellular $\text{Ca}^{2+}$ mobilization in the responses (data not shown).

Previous studies have shown that phorbol ester treatment decreases the $\text{Ca}^{2+}$ sensitivity of the CaR, whereas PKC inhibition increases it (7–9), however, the effect of down-regulating PKC expression is not shown).
CaR<sup>T888</sup> Phosphorylation

FIGURE 6. Effect of chronic phorbol ester pretreatment on CaR-induced intracellular Ca<sup>2+</sup> responses. A, intracellular Ca<sup>2+</sup> levels were measured in wtCaR-HEK and CaR<sup>T888A</sup>-HEK cells as described in the legend to Fig. 5. Cells were first pretreated for 16 h with PMA (1 μM) to down-regulate phorbol-sensitive PKC isoforms. Cell responses (minimum of 180) were categorized as before (B) and the concentration-effect curve (C) was used to estimate the EC<sub>50</sub> values of the responses. n ≥ 8.

via chronic phorbol ester pretreatment has not been reported in this context. Here we found that in wild-type CaR-HEK cells, chronic PMA pretreatment appears to increase the sensitivity of the receptor and elicits non-oscillatory, sustained responses in most cells (Fig. 6). In CaR<sup>T888A</sup> cells, chronic PMA pretreatment had little overall effect on Ca<sup>2+</sup> sensitivity but resulted in responses that were mostly sustained.

Next we examined the effect on CaR-induced Ca<sup>2+</sup> oscillations of a non-selective PKC inhibitor (GF109203X) compared with the effects of two conventional PKC (i.e. Ca<sup>2+</sup>-dependent)-selective inhibitors namely Go6976 and an anilino-monooiodobenzylmaleimide β-selective PKC inhibitor (15). Addition of GF109203X to cells exhibiting Ca<sup>2+</sup><sub>i</sub> oscillations produces sustained responses in most (≥80%) of the cells (Fig. 7). Where cells exhibiting oscillations were first treated with either 300 nM Go6976 or 500 nM PKCβ inhibitor, conversion from oscillatory to sustained Ca<sup>2+</sup> mobilization was observed in 40.1 and 27.5%, respectively, of the cells sensitive to subsequent GF109203X treatment. This concentration of PKCβ inhibitor should also inhibit PKCα and PKCγ, whereas no change in the Ca<sup>2+</sup><sub>i</sub> oscillations were seen in response to 50 nM PKCβ inhibitor that should be selective for that isozyme (15).

Next, to identify a candidate phosphatase for the dephosphorylation of CaR<sup>T888</sup>, CaR-HEK cells were pre-treated acutely with PMA (10 min) and then incubated for a further 5 min in the presence or absence of various phosphatase inhibitors. In each case, GF109203X (250 nM) was included during the 5-min incubation to prevent any further phosphorylation. In the absence of any phosphatase inhibitors, substantial dephosphorylation of CaR<sup>T888</sup> was observed within 5 min, however, in the presence of the PP1/PP2A inhibitor calyculin A (100 nM), dephosphorylation was prevented (Fig. 8A). In contrast, tautomycin (PP1 inhibitor) and FK506 (PP2B inhibitor) had little effect on CaR<sup>T888</sup> dephosphorylation (not shown). To examine this further, the colocalization of PP2A and CaR<sup>T888</sup> was investigated by dual-fluorescence immunofluorescence. CaR-HEK cells were incubated acutely in PMA and then fixed and stained with the phospho-specific anti-CaR<sup>T888</sup> antibody and a monoclonal anti-PP2A catalytic subunit antibody, followed by goat anti-rabbit (Alexa 488) and donkey anti-mouse (Alexa 594) antibodies, respectively. Merging of the resulting green (CaR<sup>T888</sup>) and red (PP2A) channels reveals partial colocalization (yellow) of the 2 antigens (Fig. 8B). Finally, to determine whether phosphatase inhibition affects CaR function, Fura-2-loaded CaR-HEK cells were exposed to 2.5 mM Ca<sup>2+</sup><sub>i</sub> to induce Ca<sup>2+</sup><sub>i</sub> oscillations and then switched to an identical buffer supplemented with 100 nM calyculin, 500 μM endothall thioanhydride, or 5 nM tautomycin. Calyculin and endothall both inhibited the Ca<sup>2+</sup><sub>i</sub> oscillations reversibly, whereas tautomycin was without effect.

To examine whether the effects of PMA and calyculin on CaR function are specific to Thr<sup>888</sup>, CaR<sup>T888A</sup>-HEK cells were stimulated with 2 mM Ca<sup>2+</sup><sub>i</sub> to elicit either oscillatory or sustained Ca<sup>2+</sup><sub>i</sub> mobilization and then cotreated with either drug. Despite the absence of Thr<sup>888</sup>, PMA still suppressed receptor-mediated Ca<sup>2+</sup><sub>i</sub> mobilization (Fig. 9A). Increasing the Ca<sup>2+</sup><sub>i</sub> concentration to 3 and then to 5 mM overcame the inhibitory effect of PMA. Interestingly, the PMA tended to ablate the Ca<sup>2+</sup><sub>i</sub> oscillations while having little effect on the rate of decline of the “sustained” responses. Similarly, cotreatment with calyculin ablated CaR<sup>T888A</sup>-induced Ca<sup>2+</sup><sub>i</sub> oscillations but had little effect on cells exhibiting a sustained response (Fig. 9B). Following the removal of calyculin, 5 mM Ca<sup>2+</sup><sub>i</sub> elicited a potent response in all cells demonstrating their continued viability.

Finally, bovine parathyroid cells were incubated in the absence or presence of PMA/calyculin, fixed, and then examined for their CaR<sup>T888</sup> content as before. The effect of PMA/calyculin cotreatment was to increase endogenous CaR<sup>T888</sup> phosphorylation in the parathyroid cells (Fig. 10A). In addition, in Fura-2-loaded bovine parathyroid cells, the rise in [Ca<sup>2+</sup>]<sub>i</sub> elicited by increasing [Ca<sup>2+</sup>]<sub>i</sub> from 0.5 to 2 mM, was suppressed by cotreatment with PMA (Fig. 10B).
**DISCUSSION**

Given the key role of the CaR in controlling extracellular calcium homeostasis, the regulation of its function has important consequences for whole animal physiology (1, 2). A role for PKC-mediated phosphorylation in the regulation of CaR activity has been proposed before, based on the use of pharmacologic modulators of PKC activity and the expression of mutant receptors lacking certain PKC consensus sites (3–5, 7–9). How- ever, to prove conclusively that CaRT888 can be phosphorylated in vivo, it has been proposed before, based on the use of pharmaco-

**HCRT888 Phosphorylation**

**FIGURE 7. Effect of PKC inhibition on CaR-induced intracellular Ca\(^{2+}\) responses.** A, intracellular Ca\(^{2+}\) levels were measured in wtCaR-HEK as described in the legend to Fig. 5. Ca\(^{2+}\) oscillations (induced by 2.5 mM Ca\(^{2+}\) \(_{\text{i}}\)) were unaffected by sham changes in solution (indicated by the downward arrow) but were converted to sustained responses by GF109203X (250 nM). B, cotreatment with the conventional PKC-selective inhibitor G66976 (300 nM) converted oscillations to sustained responses in 40% of the cells (as represented by Cell 1) but not in the remainder (Cell 2). C, cotreatment with the PKC\(\beta\)-selective inhibitor (500 nM) converted oscillations to sustained responses in 28% of the cells (as represented by Cell 1) but not in the remainder (Cell 2). Data are from a minimum of 90 cells from three independent experiments.

**FIGURE 8. Effect of protein phosphatase inhibition on CaRT888 dephosphorylation and CaR-induced Ca\(^{2+}\) oscillations.** A, wtCaR-HEK cells were incubated in the absence (lane 1) or presence (lanes 2–4) of PMA (1 \(\mu\)M, 10 min) to increase basal CaRT888 phosphorylation and either lysed immediately (lanes 1 and 2) or incubated for a further 5 min (lanes 3 and 4) in the presence of GF109203X (250 nM), further supplemented with (lane 4) or without (lane 3) the PP1/PP2A inhibitor calyculin A (100 nM). These cells were then lysed and processed with the earlier lysates for immunoblotting against the anti-CaRpT888 antibody (n = 4). B, PMA-stimulated CaR-HEK cells were fixed and costained with the polyclonal anti-CaRpT888 antibody and a monoclonal anti-PP2A catalytic subunit antibody, followed by goat anti-rabbit (Alexa 488) and donkey anti-mouse (Alexa 594) antibodies. Merging of the resulting green (CaRpT888) and red (PP2A) signal reveals partial colocalization (yellow) of the two antigens. C, Fura-2-loaded CaR-HEK cells were exposed to 2.5 mM Ca\(^{2+}\) \(_{\text{i}}\) and then switched to an identical buffer supplemented with 100 nM calyculin A, 500 \(\mu\)M to 1 mM endothall, or 5 mM tautomy-

antibody (ADD) recognizing total CaR detected the same bands as above in immunoprecipitates pulled-down using the phosphospecific antibody, whereas no anti-CaRP\(^{\text{T888}}\) immunoreactivity was observed in empty vector-transfected HEK cells. Together, these data demonstrate that the anti-CaRP\(^{\text{T888}}\) antibody detects CaR as opposed to an endogenous HEK cell protein.

Next, ablation of the signal with the phosphorylated immunizing peptide but not the non-phosphorylated peptide demonstrates the phosphospecificity of the CaRP\(^{\text{T888}}\) antibody. This is supported by the observation that cells stably expressing CaRP\(^{\text{T888}}\)A exhibited no immunoreactivity with anti-CaRP\(^{\text{T888}}\) but normal immunoreactivity with the ADD monoclonal antibody.

Having validated the antibody and demonstrated phorbol-sensitive CaRP\(^{\text{T888}}\) phosphorylation, it was then possible to demon-

strate that acute calcimimetic treatment elicited CaRP\(^{\text{T888}}\) phosphorylation in a stereoselective manner. This response was mimicked by an additional increase in Ca\(^{2+}\) concentration, an effect that was inhibited by cotreatment with the calcilytic NPS-89636 (11, 16, 17). Therefore, CaR activation elicits feedback phosphorylation of its own intracellular domain residue Thr\(^{888}\). As CaRP\(^{\text{T888}}\) phosphorylation could be induced by
CaR<sup>T888</sup> Phosphorylation

FIGURE 9. Effect of phorbol ester and calyculin treatment on CaR<sup>T888</sup>-induced intracellular Ca<sup>2+</sup> responses. A, intracellular Ca<sup>2+</sup> levels were measured in CaR<sup>T888</sup>-HEK cells in buffer containing 0.5 mM Ca<sup>2+</sup> (unless otherwise stated) as described in the legend to Fig. 5. Increasing Ca<sup>2+</sup> concentrations to 2 mM elicited oscillatory Ca<sup>2+</sup> mobilization in some cells (Cell 1) and sustained responses in others (Cell 2). After 6 min, PMA (1 μM) was added and later the Ca<sup>2+</sup> concentration was increased further (to 3 and then 5 mM) in the continued presence of PMA. Similar responses were seen with 8 separate coverslips (≥40 cells). B, CaR<sup>T888</sup>-HEK cells were stimulated with 2 mM Ca<sup>2+</sup> to induce oscillatory (Cell 1) or sustained (Cell 2) responses as before, prior to the addition of 100 nM calyculin for 4 min. Cells were allowed to recover for 3 min in 2 mM Ca<sup>2+</sup> and then treated with 5 mM Ca<sup>2+</sup> to demonstrate that the cells were still responsive. Similar responses were observed with three separate coverslips (≥25 cells).

acute PMA treatment and inhibited by GF109203X it is highly likely that the phosphorylation is PKC-dependent.

CaR<sup>T888</sup> immunoreactivity was observed in a 160-kDa band, most likely the fully mature receptor, and a 140-kDa band, presumably to be the high mannose, core-glycosylated protein. Whereas feedback phosphorylation of the mature, membrane-localized receptor could regulate receptor function or Ca<sup>2+</sup> oscillation generation/maintenance, it is less clear what purpose phosphorylating the immature receptor would serve. For example, it could regulate the maturation or forward trafficking of the CaR to the membrane. It will be interesting to determine whether under certain conditions, phosphorylation of the two bands can occur differentially, for example, by different PKC isoforms or with non-identical time courses. Thus, the current study establishes that PKC-mediated phosphorylation of the mature CaR can occur, although possibly in addition to phosphorylation of intracellular or immature receptors.

In contrast to a previous report (9), we did not find that CaR<sup>T888</sup> produced exclusively sustained Ca<sup>2+</sup> responses. Indeed, in 2 mM Ca<sup>2+</sup> more cells exhibited oscillations than sustained responses, although in 3 mM Ca<sup>2+</sup> most cells did exhibit sustained responses similar to the data reported previously (9). Nevertheless, even in 3 mM Ca<sup>2+</sup>, some CaR<sup>T888</sup>-HEK cells still exhibited oscillations and this should not be possible if dynamic changes in CaR<sup>T888</sup> phosphorylation account exclusively for Ca<sup>2+</sup> oscillations. It should be noted, however, that the oscillations induced by CaR<sup>T888</sup> here are much slower than those elicited by the wild-type CaR. The reason for the apparent discrepancy between the two studies is unclear. In the previous study (9), transient transfection was employed, whereas here we studied HEK cells stably expressing CaR<sup>T888</sup>. Given the heightened sensitivity of CaR<sup>T888</sup> it is possible that in generating the stable clone, the most responsive cells died and thus slightly less responsive cells were selected. In support of this, we noted during the selection process that the culture of CaR<sup>T888</sup>-HEK cells in Dulbecco’s modified Eagle’s medium (containing 1.8 mM CaCl<sub>2</sub>) resulted in substantial cell death necessitating the use of RPMI media instead (data not shown).

In CaR<sup>T888</sup>-HEK cells stimulated with 2 mM Ca<sup>2+</sup>, the addition of either PMA or calyculin had little effect on the sustained responses, but instead inhibited Ca<sup>2+</sup> oscillations. Increasing the Ca<sup>2+</sup> concentration, in the continued presence of PMA, overcame the inhibitory effect of the phorbol ester. Because PMA and calyculin act to increase or sustain Ser/Thr phosphorylation, respectively, these data could be explained by a PKC-mediated decrease in CaR sensitivity that occurs despite the absence of Thr<sup>T888</sup>. If true, these data could suggest that there is a further signaling determinant within CaR or its associated signaling machinery for the establishment and maintenance of Ca<sup>2+</sup> oscillations. In support of this is the fact that chronic PMA pretreatment virtually abolished Ca<sup>2+</sup> oscillations in...
CaR<sup>T888</sup> HEK cells (>98%) and did completely abolish oscillations in wild-type CaR-HEK cells. In the current study, chronic PMA pretreatment elicited heightened Ca<sup>2+</sup><sub>i</sub> sensitivity in wild-type CaR-HEK cells in a manner similar to the effects of PKC inhibitors published previously (7, 9). Chronic PMA pretreatment most likely down-regulates conventional and novel PKC isoforms (18) and PKCα and -ε have been reported to be activated upon CaR stimulation in parathyroid and CaR-HEK cells (19). These data are consistent with the previous observation (20) that CaR-induced inositol 1,4,5-trisphosphate formation was heightened in bovine parathyroid cells pre-exposed overnight to PMA (1 μM). However, in that study the Ca<sup>2+</sup><sub>i</sub>-induced Ca<sup>2+</sup><sub>i</sub> mobilization dose-response curve was not leftward shifted and neither was the suppressive effect of 2 mM Ca<sup>2+</sup><sub>i</sub> on PTH secretion significantly enhanced, although its inhibitory effect may have already been close to maximal under those conditions (20). PKC inhibitors selective for the conventional PKC isoforms partially mimicked the effect of the non-selective PKC inhibitor GF109203X in converting oscillatory Ca<sup>2+</sup><sub>i</sub> responses into sustained responses, albeit in no more than 40% of the cells. Thus, together with the data from the chronic PMA pretreatment experiments, these data could suggest that a combination of conventional and novel PKC isoforms contribute to CaR regulation.

Spontaneous dephosphorylation of CaR<sup>T888</sup> occurred in the 5 min following a 10-min PMA pretreatment indicating the presence of an active phosphatase at the CaR intracellular domain. As this effect was largely unaltered by selective inhibitors of the phosphatases PP1 and PP2B, but ablated by the PP1/PP2A inhibitor calyculin (21) and the PP2A-selective inhibitor endothall, this suggests that PP2A, or at least a calyculin-sensitive phosphatase, is responsible for this dephosphorylation. Indeed, calyculin decreased CaR-induced oscillation frequency and in some cells suppressed them entirely, consistent with the idea that CaR<sup>T888</sup> phosphorylation is inhibitory to Ca<sup>2+</sup><sub>i</sub> mobilization. Larger concentrations of calyculin gave much greater inhibitory effects (not shown). However, these cells also tended to detach rapidly from the coverslip as described for other cell types (22, 23). Thus, only experiments in which normal oscillations were recoverable following the removal of calyculin are presented. The inhibitory effect of calyculin was mimicked by 500 μM endothall thioanhydride, a protein phosphatase inhibitor (24) that exhibits greater selectivity for PP2A over PP1 (IC<sub>50</sub> PP2A, 90 nM; PP1, 5 μM). In contrast, the PP1-selective inhibitor tautomycin (5 nM) was without effect (IC<sub>50</sub> PP1, 1 nM; PP2A 10 nM). In further support of the involvement of PP2A in CaR<sup>T888</sup> dephosphorylation, the catalytic domain of PP2A colocalized, partially, with CaR<sup>T888</sup>. Together, these data implicate a calyculin-sensitive phosphatase, most likely PP2A, as the phosphatase responsible for CaR<sup>T888</sup> dephosphorylation. Thus it will be interesting to investigate CaR signaling in cells underexpressing or overexpressing the various protein phosphatases.

Finally, anti-CaR<sup>T888</sup> immunoreactivity was also observed in bovine parathyroid cells treated with PMA in the presence of calyculin indicating the potential utility of this antibody for studying CaR phosphorylation in endogenous expression systems. For example, the antibody could be used to examine whether the loss of parathyroid Ca<sup>2+</sup><sub>i</sub> sensitivity seen in conditions such as renal disease or aging involves CaR phosphorylation as opposed to down-regulation. Also, it could be used to test whether circadian changes in PTH secretion might be explained by changes in CaR<sup>T888</sup> phosphorylation and thus Ca<sup>2+</sup><sub>i</sub> sensitivity, over the course of the day.

Together these data confirm residue Thr<sup>888</sup> plays a predominant role in the regulation of Ca<sup>2+</sup><sub>i</sub> oscillations but that a further PKC-dependent mechanism could be involved, perhaps even a second phosphorylation site. In addition, we demonstrate for the first time that CaR activation induces feedback phosphorylation of the intracellular domain residue Thr<sup>888</sup> and that this residue is a substrate for a calyculin-sensitive protein phosphatase.

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