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Research Article

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Calcium-Sensing Receptors Control CYP27B1-Luciferase Expression: Transcriptional and Posttranscriptional Mechanisms

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Abbreviations: $1{,}25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3, calcitriol; 1αOHase, 25-hydroxyvitamin D 1α-hydroxylase; ANOVA, analysis of variance; BSA, bovine serum albumin; $Ca^{2+}{}_o$, extracellular Ca^{2+}; CaSR, calcium-sensing receptor; DMEM, Dulbecco's Modified Eagle Medium; ERK, extracellular regulated protein kinase; FBS, fetal bovine serum; FGF23, fibroblast growth factor 23; HRP, horseradish peroxidase; HSV, herpes simplex virus; MEK, mitogen-activated protein kinase kinase; NAM, negative allosteric modulator; PAM, positive allosteric modulator; PI-PLC, phosphoinositide-phospholipase C; PKC, protein kinase C; PTH, parathyroid hormone; RLA, Relative Luciferase Activity; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TK, tyrosine kinase.

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Abstract

25-hydroxyvitamin D 1α-hydroxylase (encoded by *CYP27B1*), which catalyzes the synthesis of 1,25-dihydroxyvitamin D_3, is subject to negative or positive modulation by extracellular Ca^{2+} ($Ca^{2+}{}_o$) depending on the tissue. However, the Ca^{2+} sensors and underlying mechanisms are unidentified. We tested whether calcium-sensing receptors (CaSRs) mediate $Ca^{2+}{}_o$-dependent control of 1α-hydroxylase using HEK-293 cells stably expressing the CaSR (HEK-CaSR cells). In HEK-CaSR cells, but not control HEK-293 cells, cotransfected with reporter genes for CYP27B1-*Photinus pyralis* (firefly) luciferase and control *Renilla* luciferase, an increase in $Ca^{2+}{}_o$ from 0.5mM to 3.0mM induced a 2- to 3-fold increase in firefly luciferase activity as well as mRNA and protein levels. Surprisingly, firefly luciferase was specifically suppressed at $Ca^{2+}{}_o \geq 5.0$mM, demonstrating biphasic $Ca^{2+}{}_o$ control. Both phases were mediated by CaSRs as revealed by positive and negative modulators. However, $Ca^{2+}{}_o$ induced simple monotonic increases in firefly luciferase and endogenous CYP27B1 mRNA levels, indicating that the inhibitory effect of high $Ca^{2+}{}_o$ was posttranscriptional. Studies with inhibitors and the CaSR C-terminal mutant T888A identified roles for protein kinase C (PKC), phosphorylation of T888, and extracellular regulated protein kinase $(ERK)_{1/2}$ in high $Ca^{2+}{}_o$-dependent suppression of firefly luciferase. Blockade

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of both PKC and $ERK_{1/2}$ abolished $Ca^{2+}{}_o$-stimulated firefly luciferase, demonstrating that either PKC or $ERK_{1/2}$ is sufficient to stimulate the CYP27B1 promoter. A key CCAAT box (–74 bp to –68 bp), which is regulated downstream of PKC and $ERK_{1/2}$, was required for both basal transcription and $Ca^{2+}{}_o$-mediated transcriptional upregulation. The CaSR mediates $Ca^{2+}{}_o$-dependent transcriptional upregulation of 1α-hydroxylase and an additional CaSR-mediated mechanism is identified by which $Ca^{2+}{}_o$ can promote luciferase and possibly 1α-hydroxylase breakdown.

Key Words: calcium-sensing receptor, CYP27B1, 1αOHase, PKC, Thr-888 phosphorylation, ERK1/2, transcription, protein synthesis, protein breakdown

The mitochondrial membrane enzyme 25-hydroxyvitamin D 1α-hydroxylase (1αOHase), which is encoded by the *CYP27B1* gene, catalyzes the synthesis of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; calcitriol). The hormonal form of $1,25(OH)_2D_3$, which is generated in the renal proximal tubule [1], acts systemically to promote intestinal calcium and inorganic phosphate absorption and thereby plays a key role in whole-body mineral metabolism (reviews: [2-5]). Consistent with this role, mutations of *CYP27B1* that impair the structure and/or function of 1αOHase cause vitamin D-dependent rickets type-1 (VDDR-I), typified by low plasma ionized calcium and phosphate concentrations, and mineralization defects [6, 7] and many but not all of the effects of CYP27B1 deficiency are restored by calcium- and phosphate-rich rescue diets (review: [8]).

Thus, $1,25(OH)_2D_3$ generated locally in extrarenal tissues via 1αOHase encoded by the same *CYP27B1* gene, acts via vitamin D receptor–dependent and vitamin D receptor–independent paracrine, autocrine, and even intracrine mechanisms to modulate cellular responses. While the full significance of these effects and the nature of their local control remains unclear, they include inhibition of parathyroid hormone transcription [9-11], negative control of parathyroid hyperplasia [12], phenotypic modulation of growth plate chondrocytes [13], basal levels of osteoblastic bone formation [12], and immunomodulatory and bactericidal actions in cells of the monocyte/macrophage lineage (review: [14]).

1αOHase activity is modulated differentially by hormones, cytokines, and nutrients according to the cell type in which it is expressed. In renal proximal tubule epithelial cells, key modulators include the hormones parathyroid hormone (PTH) and calcitonin, which are stimulatory [15], and $1,25(OH)_2D_3$ [15] and fibroblast growth factor 23 (FGF23) [16-19], which are inhibitory. These hormones act, at least in part, on the promoter region of the *CYP27B1* gene via receptor-dependent signaling mechanisms. Thus, PTH [20-23]) and calcitonin [24] increase and $1,25(OH)_2D_3$ [21, 25] and FGF23 [19] suppress CYP27B1 mRNA levels.

Two key nutrients that have been identified previously as modulators of CYP27B1 and its encoded enzyme 1αOHase include calcium [26-28] and inorganic phosphate [29]. These effects operate in part systemically via $Ca^{2+}{}_o$- (review: [30]) and inorganic phosphate- [31] dependent control of PTH secretion and by phosphate-dependent control of FGF23 secretion from osteocytes (review: [4]). They also operate locally, that is, directly on cells of the proximal tubule, parathyroid, and osteoblast lineage. Thus $Ca^{2+}{}_o$, independent of its effects on PTH [27], directly suppresses 1αOHase activity in the proximal tubule [28] but stimulates it in other sites, including parathyroid cells [32] and osteoblasts [33]. Whether the inhibitory effect of $Ca^{2+}{}_o$ on 1αOHase activity in the proximal tubule and stimulatory effect of $Ca^{2+}{}_o$ on 1αOHase activity in extrarenal tissues are mediated by a plasma membrane receptor and via CYP27B1 transcription or some other mechanism have not been elucidated.

The extracellular calcium-sensing receptor (CaSR) is a widely expressed Ca^{2+}-sensor responsible for mediating diverse $Ca^{2+}{}_o$-dependent effects. In the parathyroid, the CaSR mediates $Ca^{2+}{}_o$-dependent inhibition of PTH synthesis and secretion (review: [30]). In the renal proximal tubule, where it is expressed but at relatively low levels [34] it mediates $Ca^{2+}{}_o$-induced disinhibition of the phosphaturic action of PTH [35] and may thus also impair PTH-induced upregulation of 1αOHase. In osteoblasts and chondrocytes, the CaSR promotes cellular maturation leading to enhanced matrix synthesis and mineralization ([36]; review: [37]), at least in part via a newly described signaling pathway dependent on phospho-Akt [38, 39]. In addition, tissue-specific knockouts of the floxed CaSR in chondrocytes (targeted via the type-II collagen promoter) and in osteoblasts (targeted via the type-I collagen promoter) resulted in major developmental abnormalities and growth retardation in mice [40]. These considerations led us to hypothesize

that one or more actions of $Ca^{2+}{}_{o}$ on $1{,}25(OH)_2D_3$ synthesis via 1αOHase, including suppressed synthesis in the renal proximal tubule and/or stimulated synthesis in extrarenal tissues such as the parathyroid and skeletal osteoblasts, might be mediated by the CaSR.

Consistent with our hypothesis, the CaSR is expressed in proximal tubule epithelial cells ([34]; review [41]), parathyroid cells [42], growth plate chondrocytes, and osteoblasts (review: [43]). However, none of the commonly used cell models from these tissues appeared suitable for a test of our hypothesis since they are difficult to maintain in primary culture and to transfect, and they exhibit downregulation of the CaSR ([44]) and other key receptors (eg, PTH1R in proximal tubule cells; [45]). We therefore focused on a cell type that expresses the CaSR stably and robustly, that can be readily transfected with constructs suitable for evaluating CaSR-mediated control of CYP27B1 expression, and for which there is an appropriate CaSR-null control.

For these reasons, in the present study we investigated whether the CaSR mediates $Ca^{2+}{}_{o}$-dependent control of CYP27B1 promoter–dependent luciferase expression using a well-defined model cell type, HEK-293 cells that stably express the CaSR (HEK-CaSR cells). HEK-293 cells were previously used to demonstrate the inhibition of CYP27B1 promoter–dependent luciferase expression by FGF-23 [19] and to demonstrate CaSR-mediated upregulation of 2 immediate early genes, Egr-1 and AP-1 [46]. To facilitate our analyses, HEK-CaSR cells were transfected with a CYP27B1 promoter-*Photinus pyralis* firefly luciferase reporter gene and a control, herpes simplex virus (HSV) thymidine kinase (TK) promoter-*Renilla* luciferase construct as described previously [47].

To our surprise, we found that the CaSR mediates a complex, biphasic $Ca^{2+}{}_{o}$ concentration–dependent response. At submaximal $Ca^{2+}{}_{o}$ concentrations (0.5-3.0mM), the CaSR mediated transcriptional upregulation of CYP27B1-firefly luciferase. However, at maximal $Ca^{2+}{}_{o}$ concentrations (≥5.0mM), the CaSR mediated a posttranscriptional downregulation of luciferase protein levels. Although luciferase and 1αOHase are clearly distinct proteins, the results indicate that at high $Ca^{2+}{}_{o}$ concentrations the CaSR mediates enhanced breakdown of selected proteins with potential significance beyond the model HEK-293 cell/luciferase system described here.

Materials and Methods

Cell Culture

HEK-293 cells, or HEK-293 cells that stably express wild-type human CaSR (HEK-CaSR) or a T888A CaSR mutant (HEK-CaSR^{T888A}) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Bovogen Biologicals SFBS-AU), and 0.5% (v/v) penicillin-streptomycin in a humidified, 37 °C, 5% CO_2 incubator. Cells were grown until 80% confluency, detached with 0.25% trypsin-EDTA (Gibco 11995), seeded into culture plates at ~30% confluency, and incubated for > 6 hours to adhere.

CYP27B1 Promoter-Luciferase Constructs

Plasmids containing the firefly luciferase reporter gene under the control of the human CYP27B1 promoter (CYP27B1-luciferase) and internal control *Renilla* luciferase reporter gene under the thymidine kinase (TK) promoter (pRL-TK; Promega) were developed previously [47]. CYP27B1-luciferase reporter constructs contain −1501 or −305 base pairs (bp) of the 5′ flanking region, and +42 bp of the 5′ untranslated region from the human *CYP27B1* gene. These sequences had been cloned into the pGL3-Basic Vector, upstream of the firefly luciferase reporter gene (Promega), as described in (22).

Site-Directed Mutagenesis and PCR Amplification

Mutations of putative response elements were introduced into the CYP27B1-luciferase reporter constructs by polymerase chain reaction (PCR), using 25- to 30-base long forward and reverse primers. PCR was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA) according to the manufacturer's instructions, with DMSO included to reduce secondary structure formation. Where possible, nucleotide changes were selected based on those used in (22). A table of primers used in site-directed mutagenesis is provided in Supplemental Table 1 [48].

Truncations of the CYP2B1 promoter were generated by PCR amplification with MyTaq Polymerase (Bioline) using primers designed to anneal to the −1501 bp CYP27B1-luciferase construct at −100 and −200 bp, with an *Asc*I restriction site included on the 5′ ends. The PCR product was digested with 5 U *Dpn*I and 5 U *Asc*I, and ligated into plasmid using 400 U T4 DNA ligase according to manufacturer's instructions to generate the −102 and −200 bp CYP27B1 promoter-luciferase constructs. The PCR primers used to truncate the CYP27B1 promoter are provided in Supplemental Table 2 [48].

DNA samples were sequenced at the Australian Genome Research Facility (Brisbane, Australia) using primers described in Supplemental Table 3 [48]. The retrieved sequences were aligned against known sequences using Serial Cloner, version 2.6.

Transfection of HEK-CaSR Cells With Plasmid DNA

HEK-CaSR cells plated into 48-well plates were incubated in Ca^{2+} free DMEM (Gibco) supplemented with 0.5mM Ca^{2+}_o and 10% (v/v) FBS (Bovogen Biologicals SFBS-AU), and transiently transfected with luciferase constructs using X-tremeGENE HP DNA Transfection Reagent (Sigma Aldrich #06366236001) according to manufacturer's instructions using a 4:1 ratio of transfection reagent to DNA and incubated for 48 hours.

Cell Incubations

Cells were incubated for 24 hours in Ca^{2+}-free DMEM (Gibco 21068) supplemented with 0.2% bovine serum albumin (BSA; Sigma Aldrich A9576) and adjusted for Ca^{2+}_o using $CaCl_2$. Cinacalcet and NPS-2143 (kindly provided by the Leach lab; The Monash Institute of Pharmaceutical Sciences) were used as positive and negative CaSR modulators respectively. To investigate the roles of signaling pathways, inhibitors were used to pretreat cells for 1 hour prior to 24-hour incubation. Following incubation the cells were washed once in chilled phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4) and lysates prepared as required for Dual-Luciferase Reporter (DLR) Assay System, quantitative real-time reverse transcriptase (RT)-PCR, or Western blotting.

Dual-Luciferase Reporter Assays

Cell lysates were prepared, and *P. pyralis* (firefly) and *Renilla* luciferase bioluminescence were generated using substrate solutions provided in the DLR Assay System (Promega E1910) according to the manufacturer's instructions. Bioluminescence measurements were normalized for background (untransfected cell lysates) and expressed either as raw bioluminescence counts or as Relative Luciferase Activities, that is, as fold changes of firefly luciferase with respect to *Renilla* luciferase.

Quantitative Real-Time RT-PCR

RNA was isolated using TRI Reagent (Sigma Aldrich T9424), digested using DNase I (Sigma Aldrich AMPD1) to remove contaminating DNA, then reverse transcribed using Tetro cDNA Synthesis kit (Bioline BIO-65042) according to manufacturer's instructions. Real-time PCR analyses were performed on diluted cDNA using the SensiFAST SYBR No-ROX Kit (Bioline BIO-98002) after identifying the linear range for cDNA dilutions and assessing melt temperature and amplicon sizes.

Relative expression levels were calculated by the $2^{-\Delta\Delta C_T}$ method using the cycle threshold (C_T) of the gene of interest and the housekeeping gene (RNA polymerase II) [49] obtained from Corbett Rotor-Gene 6000 Series Software 1.7.

Western Blotting—Antibodies

The following antibodies were used: Anti-firefly luciferase (rabbit polyclonal; Sigma #L0159; [50]); Anti-GAPDH (rabbit monoclonal; Cell Signaling Technology #2118; [51]); Anti-rabbit IgG-horseradish peroxidase [HRP] conjugated (goat polyclonal, Abcam #97051; [52]); Anti-CaSR *phospho*T888 (rabbit polyclonal, custom-generated and affinity purified; [53]); Anti-CaSR total (mouse monoclonal, Thermo-Fisher #MA1-934; [54]); Anti-rabbit Ig-HRP conjugated (goat polyclonal, Agilent #P0448; [55]); Anti-mouse IgG-HRP conjugated (horse polyclonal, Cell Signaling Technology #7076; [56]).

Western Blotting—Procedures

Cells were lysed using a modified radioimmunoprecipitation assay (RIPA) buffer (25mM Tris-HCl, pH 7.6, 150mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1× Protease Inhibitor Cocktail [Cell Signaling Technology]) and triturated 30 times through a 21G needle. In some experiments the RIPA-like lysis buffer contained 12mM HEPES (pH 7.6), 300mM mannitol, 1mM EGTA, 1mM EDTA, 1% (v/v) Triton-X 100, 0.1% (w/v) SDS, 1mM NaF, 100μM sodium vanadate, 250μM sodium pyrophosphate, 1.25μM pepstatin, 4μM leupeptin, and 4.8μM PMSF. Protein samples were run through SDS-PAGE and transferred electrophoretically to 0.45-μm nitrocellulose membranes (Bio-Rad). Membranes were incubated in solutions containing antibodies against firefly luciferase or GAPDH according to manufacturers' instructions, or against anti-phosphoCaSR^{T888} [57], and then HRP-conjugated secondary antibodies as listed above according to the species in which the primary antibodies were raised. The reaction catalyzed by HRP was initiated using Pierce ECL Western Blotting Substrate (Life Technologies Australia). Bands were visualized and densitometry analyses performed on a ChemiDoc MP Imaging System and Image Lab Software (Bio-Rad).

Statistical Analyses

The results are routinely expressed as means ± SEM. Statistical analyses were performed using 1-way analysis of variance (ANOVA) for Ca^{2+}_o concentration–response experiments, and 2-way ANOVA for studies involving CaSR modulators

and inhibitors of signaling, followed by Sidak's, Dunnett's, or Tukey's multiple comparisons test. $P < 0.05$ was used to assign statistical significance (GraphPad Prism 7.0).

Results

Effect of Elevated $Ca^{2+}{}_o$ on Luciferase Activity

HEK-CaSR cells or control HEK-293 cells were transiently cotransfected with (i) firefly luciferase under the control of the CYP27B1 1501 bp promoter; and (ii) *Renilla* luciferase under the control of a constitutive HSV-TK promoter. The transfected cells were then exposed to various $Ca^{2+}{}_o$ concentrations to determine the impact of CaSR activation on firefly luciferase, as a measure of CYP27B1 promoter function, and control *Renilla* luciferase activity.

In 3 initial experiments we incubated transfected HEK-CaSR cells with 3 different $Ca^{2+}{}_o$ concentrations, 0.5mM, 3.0mM, and 5.0mM and analyzed both firefly and *Renilla* luciferase activities at 2, 4, 8, and 24 hours (Fig. 1). At 0.5mM $Ca^{2+}{}_o$, there was no change in the baseline level of either firefly or *Renilla* luciferase at any of the time points over the 24-hour period of the experiment. However, at 3.0mM $Ca^{2+}{}_o$, we observed a linear increase in firefly luciferase, but not *Renilla* luciferase, that was apparent after 4 hours and reached a level of around 2- to 4-fold of baseline after 24 hours (Fig. 1A). There was no significant change in *Renilla* luciferase activity from 2 to 24 hours at 3.0mM $Ca^{2+}{}_o$. Interestingly, at 5.0mM $Ca^{2+}{}_o$, we observed no significant changes in either firefly or *Renilla* luciferase at any of the time points studied over the course of the 24-hour period. Thus, we observed a striking biphasic $Ca^{2+}{}_o$ concentration response for firefly luciferase, but not *Renilla* luciferase, which was maximal at 3.0mM $Ca^{2+}{}_o$ (Fig. 1A) and dependent on the CYP27B1 promoter immediately upstream of the firefly luciferase gene. These effects were also apparent when they were expressed as Relative Luciferase Activity (firefly luciferase/*Renilla* luciferase; Fig. 1B) thereby using *Renilla* luciferase as a control for variations in transfection efficiency between experiments. Based on these effects we routinely expressed the data as Relative Luciferase Activity (RLA) and selected 24 hours as the standard incubation time.

We confirmed and extended these observations in subsequent experiments. Thus, HEK-CaSR cells elevated $Ca^{2+}{}_o$ (3.0mM) stimulated firefly luciferase activity by 3- to 4-fold over 24 hours but had little or no effect on *Renilla* luciferase (Fig. 2A). However, as described above, HEK-CaSR cells exposed to 5.0mM or 8.0mM $Ca^{2+}{}_o$ for 24 hours exhibited markedly reduced firefly luciferase activity when compared with the peak response at 3.0mM $Ca^{2+}{}_o$ ($P < 0.05$), lying close to the baseline level observed at 0.5mM $Ca^{2+}{}_o$ (Fig. 2A). No effect, however, was observed on *Renilla* luciferase.

When the results in Fig. 2A were expressed as RLA, elevated $Ca^{2+}{}_o$ (3.0mM) induced a 2- to 3-fold increase over 24 hours when compared with control $Ca^{2+}{}_o$ (0.5mM) in HEK-CaSR cells (Fig. 2B) but not in control HEK-293 cells that do not express the CaSR (Fig. 2C) and, consistent with the results in Fig. 2A, HEK-CaSR cells exposed to 5.0mM or 8.0mM $Ca^{2+}{}_o$ for 24 hours exhibited markedly reduced RLA when compared with the peak response at 3.0mM $Ca^{2+}{}_o$ ($P < 0.05$). These findings indicate that an increase in $Ca^{2+}{}_o$ from 0.5mM to 3.0mM activated a CaSR-dependent stimulatory signaling pathway upstream of firefly luciferase expression but that a further increase in $Ca^{2+}{}_o$ to 5.0mM or higher concentrations activated an inhibitory signaling pathway. Thus, the findings point to the existence of a biphasic response in the $Ca^{2+}{}_o$ range from 0.5mM to 5.0mM,

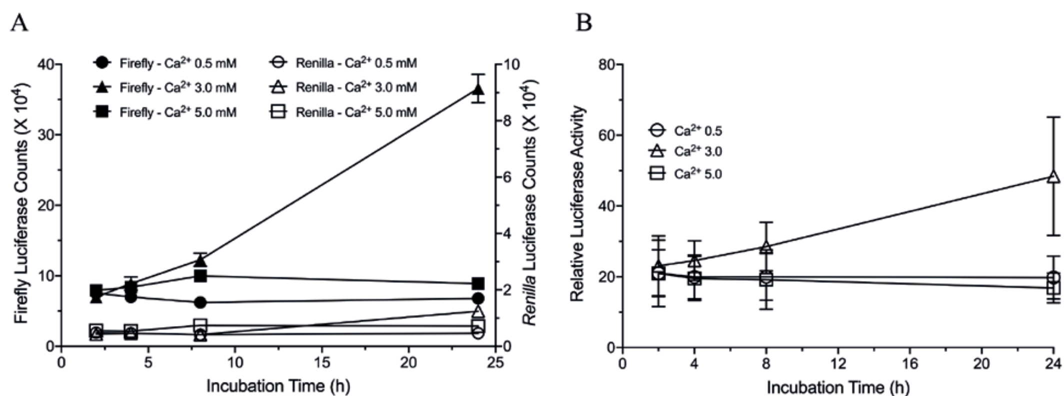

Figure 1. $Ca^{2+}{}_o$ concentration and time dependencies of firefly and *Renilla* luciferase activity in cotransfected HEK-CaSR cells. HEK-CaSR cells were transiently cotransfected with full-length (1501 bp) CYP27B1-firefly luciferase reporter gene and the TK-*Renilla* luciferase control gene, then incubated at various $Ca^{2+}{}_o$ concentrations as shown for various times from 2 to 24 hours prior to analysis for both firefly and *Renilla* luciferases. In (A) the data from a single experiment performed in triplicate are expressed as separate firefly and *Renilla* luciferase activities. Similar results were obtained in 2 further experiments. In (B) the data were obtained in 3 experiments and are expressed as Relative Luciferase Activities.

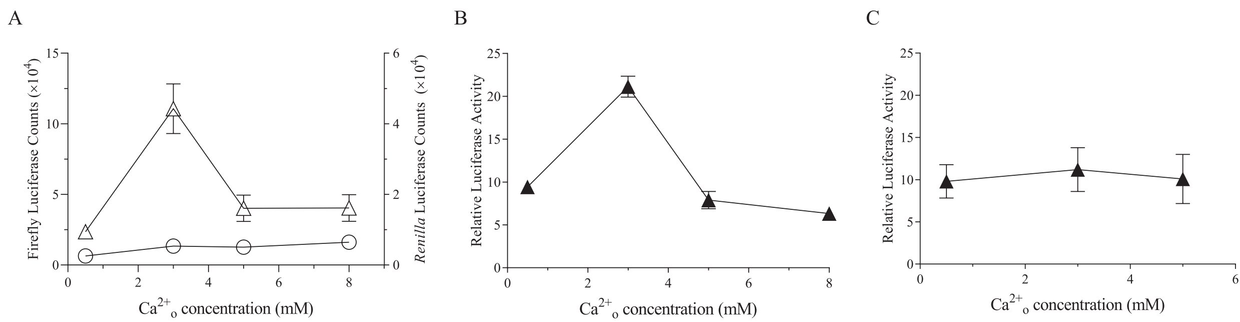

Figure 2. Biphasic Ca^{2+}_o concentration–dependent control of relative luciferase activity in HEK-CaSR cells cotransfected with CYP27B1–firefly luciferase and control *Renilla* luciferase constructs. HEK-CaSR and HEK-293 cells were transiently cotransfected with full-length (1501 bp) CYP27B1-firefly luciferase reporter gene and the TK-*Renilla* luciferase control gene, then incubated for 24 hours at various Ca^{2+}_o concentrations prior to analysis for both firefly and *Renilla* luciferases. The results show the impacts of Ca^{2+}_o on the activities of: (A) firefly luciferase (open triangles) and *Renilla* luciferase (open circles) in HEK-CaSR cells; (B) Relative Luciferase Activity (firefly/*Renilla*) in HEK-CaSR cells; and (C) Relative Luciferase Activity in HEK-293 cells. The data were obtained in 4 independent experiments for panels A and B, and in 6 independent experiments in C. Luciferase activities were corrected for the background levels obtained in untransfected cells. Some errors lie entirely within the symbols.

with a peak at approximately 3.0mM. Neither the stimulatory nor the inhibitory phases of the Ca^{2+}_o-dependent biphasic response in RLA could be detected at shorter time points ≤ 4 hours (3 independent experiments, not shown).

The CaSR Mediates Biphasic Control of Luciferase Activity

Transfected HEK-CaSR cells were next exposed to the CaSR positive allosteric modulator (PAM) cinacalcet, or the CaSR negative allosteric modulator (NAM) NPS-2143, to further investigate whether the CaSR mediates either 3.0mM Ca^{2+}_o-induced stimulation of firefly luciferase expression or 5.0mM Ca^{2+}_o-induced suppression.

Cinacalcet (1 µM) markedly enhanced RLA in cells exposed to control Ca^{2+}_o (0.5mM) for 24 hours and also suppressed RLA at 3.0mM ($P < 0.01$; Fig. 3A). However, there was no apparent change at 5.0mM (not shown). Thus, cinacalcet shifted the biphasic Ca^{2+}_o concentration–response relationship to the left, indicating that it had increased the Ca^{2+}_o sensitivity of both the stimulatory and inhibitory phases. The results are consistent with the conclusion that both phases of the response are CaSR dependent.

The CaSR negative modulator NPS-2143 (1 µM), on the other hand, appeared to right-shift the Ca^{2+}_o concentration–response curve. Thus, RLA was unchanged at the control Ca^{2+}_o of 0.5mM but significantly suppressed at Ca^{2+}_o 3.0mM ($P < 0.01$; Fig. 3B) and further increases in Ca^{2+}_o to 5.0mM or 7.0mM stimulated, rather than inhibited, RLA by around 3-fold, compared with the 0.5mM Ca^{2+}_o control ($P < 0.001$ compared with 0.5mM Ca^{2+}_o).

Similar results were obtained for firefly luciferase protein expression by Western blotting, in which band intensities increased 2.9 ± 0.4-fold at 3.0mM Ca^{2+}_o when compared with 0.5mM Ca^{2+}_o, then decreased at 5.0mM Ca^{2+}_o to levels comparable to control (Fig. 3C). Furthermore, the CaSR PAM cinacalcet shifted the biphasic response to the left, and the CaSR NAM NPS-2143 shifted it to the right. Densitometry analyses of the protein levels for the GAPDH control demonstrated that there were no significant differences between any of the treatments (not shown). These results indicate that the biphasic Ca^{2+}_o concentration–dependent changes in RLA arose from CaSR-mediated biphasic Ca^{2+}_o concentration–dependent changes in firefly luciferase protein levels.

Elevated Ca^{2+}_o Increases Luciferase mRNA Expression

We next performed quantitative RT-PCR for luciferase mRNA to investigate whether either 3.0mM Ca^{2+}_o-dependent stimulation or 5.0mM Ca^{2+}_o-dependent suppression of firefly luciferase activity was dependent on changes in firefly luciferase mRNA levels, that is, whether the changes in firefly luciferase activity were dependent on changes in the activation of the CYP27B1 promoter. As for luciferase we observed time-dependent increases in mRNA levels from 2 to 24 hours and selected 24 hours as a convenient time to undertake quantitations and comparisons. Thus, we observed increased (3.1 ± 0.5-fold) firefly luciferase mRNA levels in response to an increase in Ca^{2+}_o from 0.5mM to 3.0mM ($P < 0.01$; Fig. 4A), which resembled the observed increase in firefly luciferase enzyme activity (Figs. 1 and 2). The results are consistent with the conclusion that in HEK-CaSR cells exposed to 3.0mM Ca^{2+}_o, increases in firefly luciferase activity arose from enhanced transcription via the CYP27B1 promoter. Interestingly, unlike the observation for firefly luciferase *activity*, there was no significant

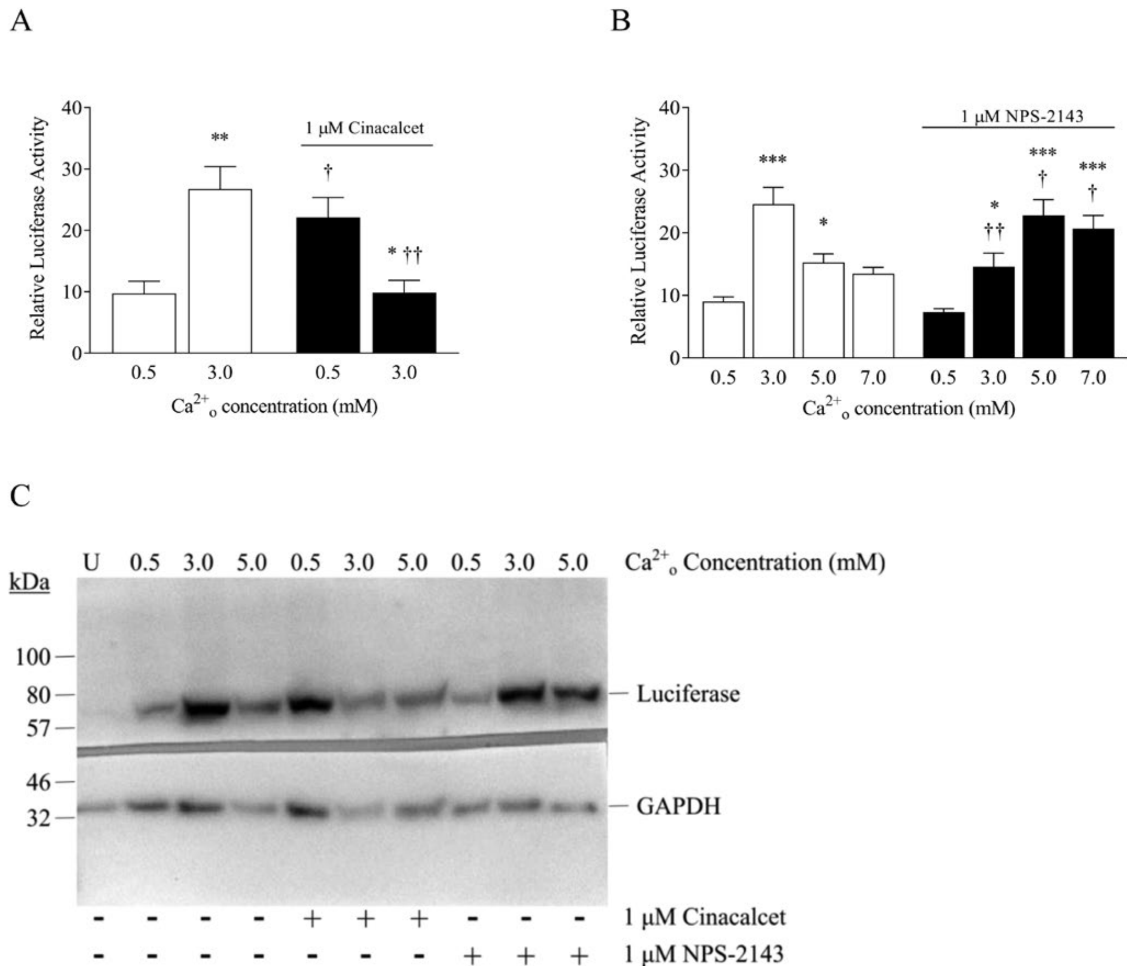

Figure 3. The CaSR mediates biphasic Ca^{2+}_o concentration–dependent control of CYP27B1 luciferase expression. Luciferase assays were performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase reporter gene under the control of the CYP27B1 −1501 bp promoter, and the *Renilla* luciferase gene control under the HSV-TK promoter. Cells were incubated for 24 hours in 0.2% BSA supplemented DMEM in the presence of various Ca^{2+}_o, in the absence or presence of (A) cinacalcet (1μM) or (B) NPS-2143 (1μM). Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with 0.5mM Ca^{2+}_o; † $P < 0.05$; †† $P < 0.01$ compared with absence of modulator. Control data are shown at the left in both panels. The data were obtained in 4 and 7 independent experiments performed in triplicate for Figures A and B, respectively. (C) HEK-CaSR cells were incubated as described for (A) and (B) and Western blotting was performed for firefly luciferase protein. A representative Western blot is shown from 3 independent experiments. Abbreviation: U, untransfected HEK-CaSR control cells.

difference in the level of firefly luciferase mRNA between that observed at 3.0mM Ca^{2+}_o and that observed at 5.0mM Ca^{2+}_o, which exhibited a 2.7 ± 0.6-fold elevation when compared with the 0.5mM Ca^{2+}_o control. The results indicate that the inhibition of firefly luciferase activity observed at 5.0mM Ca^{2+}_o when compared with 3.0mM Ca^{2+}_o did not arise from Ca^{2+}_o-dependent inhibition of transcription or loss of mRNA stability.

Ca^{2+}_o-Dependent Increase of 1αOHase mRNA is CaSR-Mediated

We next investigated whether it was possible to detect endogenous 1αOHase mRNA and protein in HEK-CaSR and HEK-293 cells and, if so, whether the levels of either mRNA or protein might be modulated by Ca^{2+}_o. The 1αOHase mRNA levels exhibited an apparent 2- to 3-fold increase at 3.0mM Ca^{2+}_o and a 4- to 6-fold increase at 5.0mM Ca^{2+}_o at 24 hours. We were unable to detect, however, any Ca^{2+}_o-dependent changes in 1αOHase protein, which was at the limit of sensitivity of the Western blot assay.

We selected 24 hours for subsequent experiments on the Ca^{2+}_o concentration dependence of 1αOHase mRNA expression over an extended Ca^{2+}_o range of 0.5mM to 7.0mM. Increasing Ca^{2+}_o from 0.5mM to 3.0mM, 5.0mM, or 7.0mM increased 1αOHase mRNA levels in HEK-CaSR cells, with a maximal 3.4 ± 0.4-fold stimulation observed at 5.0mM Ca^{2+}_o ($P < 0.001$; Fig. 4B) and no significant difference in 1αOHase mRNA expression was observed as Ca^{2+}_o was increased between 5.0 mM and

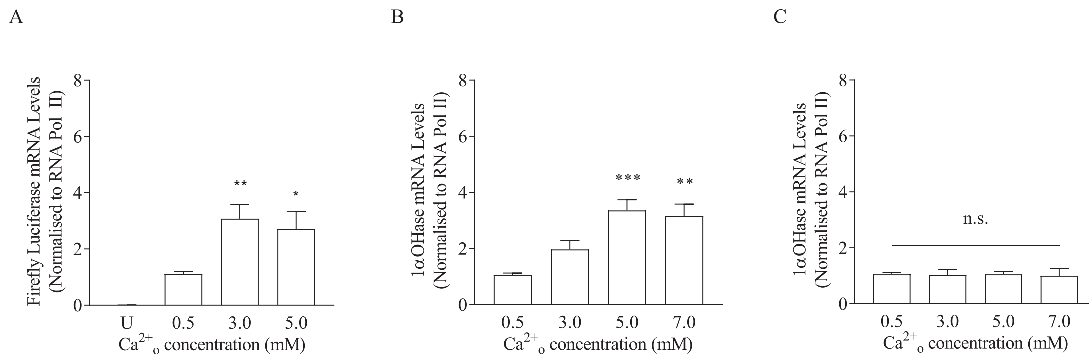

Figure 4. Ca^{2+}_o stimulated firefly luciferase and 1αOHase mRNA expression in transfected HEK-CaSR cells. Quantitative RT-PCR for (A) firefly luciferase expression was performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase reporter gene under the CYP27B1 −1501 bp promoter, and the *Renilla* luciferase gene control under the HSV-TK promoter. Cells were incubated for 24 hours in 0.2% BSA supplemented DMEM at 0.5mM, 3.0mM, or 5.0mM Ca^{2+}_o. (B) 1αOHase expression was also quantified in untransfected HEK-CaSR cells and (C) HEK-293 cells, as described, for 0.5mM, 3.0mM, 5.0mM, or 7.0mM Ca^{2+}_o. mRNA expression levels were expressed as fold change with respect to the 0.5mM Ca^{2+}_o control, relative to RNA polymerase II using the $2^{-\Delta\Delta C_T}$ method. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with 0.5mM Ca^{2+}_o-treated cells using 1-way ANOVA with Dunnett's post-test. The data in (A) were obtained in 5 independent experiments, (B) were obtained in 4 independent experiments, and (C) were obtained in 3 independent experiments, performed in duplicate.

7.0mM Ca^{2+}_o. Thus, we did not observe biphasic Ca^{2+}_o concentration–dependent control of 1αOHase mRNA expression in HEK-CaSR cells.

Ca^{2+}_o-stimulated increases in 1αOHase mRNA levels observed in HEK-CaSR cells were absent in control HEK-293 cells up to a Ca^{2+}_o of 7.0mM (Fig. 4C). Thus, the CaSR mediated Ca^{2+}_o-dependent increases in 1αOHase mRNA expression from 0.5mM to 7.0mM Ca^{2+}_o.

Effect of PKC Inhibitor on CaSR-Mediated Control of Luciferase Activity

Because we had demonstrated that the CaSR mediates (i) both the stimulatory and inhibitory control of RLA downstream of the CYP27B1 promoter as well as (ii) stimulatory control of 1αOHase mRNA levels in HEK-CaSR cells, we investigated the impacts on CYP27B1 promoter–dependent luciferase protein levels of selective inhibitors of key signaling pathways downstream of the CaSR, focusing first on PKC. Several PKC isoforms, including α and ε, are activated in response to the Ca^{2+}_o-stimulated CaSR [58, 59] and PKC is activated by either phosphoinositide-phospholipase C (PI-PLC)-dependent and/or -independent mechanisms downstream of G-protein coupled receptors (review: [60]). To investigate the role of PKC, HEK-CaSR cells were preincubated for 1 hour in the absence or presence of the potent PKC inhibitor GF109203X, a known inhibitor of PKC α, β_1, β_2, and γ [61] and the cells were then incubated for a further 24 hours in the continuing presence of GF109203X at 0.5mM, 3.0mM, or 5.0mM Ca^{2+}_o. GF109203X (0.5µM and 1µM) had no effect on basal RLA at 0.5mM Ca^{2+}_o or on 3.0mM Ca^{2+}_o-induced stimulation of RLA. Surprisingly, however, GF109203X (1µM) reversed the inhibitory effect observed at 5.0mM Ca^{2+}_o (Fig. 5A).

Role of CaSR T888 Phosphorylation

Having identified a role for PKC in CaSR-mediated inhibitory control of CYP27B1-dependent luciferase expression, we investigated whether the key PKC phosphorylation site at CaSR residue T888 might be required for the effect using HEK-293 cells that stably expressed the CaSR mutant T888A (HEK-$CaSR^{T888A}$), which disables the phosphorylation site [57, 62]. In pilot experiments, we observed an apparent loss of the inhibitory phase of the Ca^{2+}_o concentration–dependent relationship in HEK-$CaSR^{T888A}$ cells. For this reason, we performed the experiments over an extended Ca^{2+}_o range (0.5mM to 12mM).

HEK-$CaSR^{T888A}$ cells exhibited Ca^{2+}_o-stimulated firefly luciferase expression at 3.0mM that was comparable to that observed in HEK-CaSR cells, which, as noted above, express the wild-type CaSR (Fig. 5B). Furthermore, the CaSR PAM cinacalcet (1 µM) enhanced RLA at 0.5mM Ca^{2+}_o in HEK-$CaSR^{T888A}$ cells ($P < 0.05$; Fig. 5B).

However, unlike HEK-CaSR cells, 5.0mM to 12mM Ca^{2+}_o did not suppress RLA in HEK-$CaSR^{T888A}$ cells. Furthermore, cinacalcet failed to enhance the inhibitory effect of 5.0mM Ca^{2+}_o on RLA in HEK-$CaSR^{T888A}$ cells (Fig. 5B). The results indicate that CaSR-T888A supported the stimulatory phase of the Ca^{2+}_o-dependent relationship but was unable to support the inhibitory phase at higher Ca^{2+}_o concentrations (from 5.0mM to 12mM). They indicate further that PKC acting via T888 phosphorylation on the CaSR itself mediates the inhibitory effect of high Ca^{2+}_o on CYP27B1-dependent firefly luciferase expression.

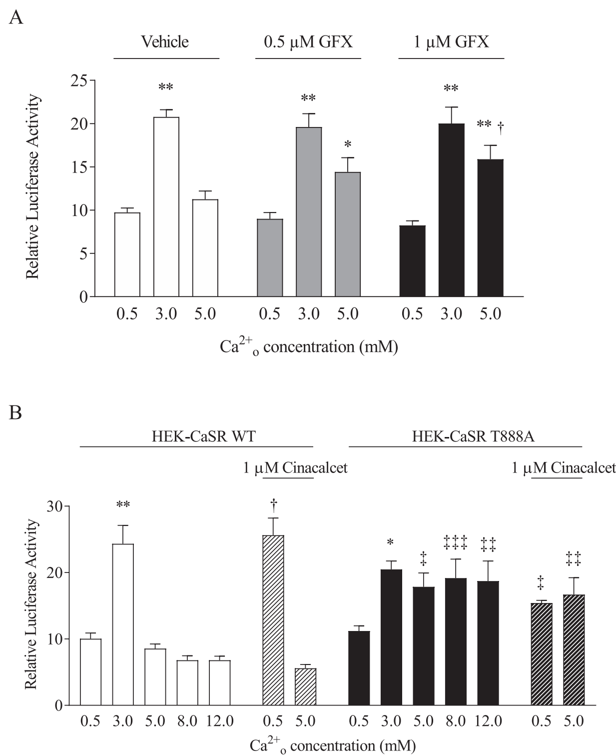

Figure 5. High Ca^{2+}_{o}-induced inhibition of CYP27B1-firefly luciferase: requirement for protein kinase C phosphorylation of CaSR C-terminus. (A) Effect of PKC inhibitor, GF109203X, on Ca^{2+}_{o}-dependent CYP27B1-firefly luciferase. Luciferase assays were performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase reporter gene under the CYP27B1 –1501 bp promoter, and the *Renilla* luciferase gene control under the HSV-TK promoter. Cells were preincubated for 1 hour in 0.2% BSA supplemented DMEM at 0.5mM Ca^{2+}_{o} in the presence of vehicle (0.1% DMSO), GF109203X (GFX; 0.5µM or 1.0µM). The Ca^{2+}_{o} concentration was then either maintained at 0.5mM or increased to 3.0mM or 5.0mM for 24 hours. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.01$; ** $P < 0.001$ compared with 0.5mM Ca^{2+}_{o}; † $P < 0.05$ compared with vehicle (0.1% DMSO). The data were obtained in 6 independent experiments performed in triplicate. (B) Effect of disabling CaSR T888 phosphorylation. Luciferase assays were performed on HEK-CaSR and HEK-$CaSR^{T888A}$ cells. Cells were transiently cotransfected with the firefly luciferase reporter gene under the CYP27B1 –1501 bp promoter, and the *Renilla* luciferase gene control under the HSV-TK promoter. Cells were incubated in 0.2% BSA supplemented DMEM in the presence of various Ca^{2+}_{o} in the absence or presence of cinacalcet for 24 hours. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.05$; ** $P < 0.001$ compared with 0.5mM Ca^{2+}_{o}; † $P < 0.001$ compared with absence of cinacalcet; ‡ $P < 0.05$; ‡‡ $P < 0.01$; ‡‡‡ $P < 0.001$ compared with wild-type CaSR. The data were obtained in 6 independent experiments performed in triplicate.

Chronically Elevated Ca^{2+}_{o} Promotes CaSR T888 Phosphorylation

The effects of various Ca^{2+}_{o} concentrations on wild-type CaSR T888 phosphorylation were assessed using an epitope-specific antibody [57] over the extended incubation period used in the present study. In response to an increase in Ca^{2+}_{o} from 0.5mM to 3.0mM or 5.0mM over 24 hours, we observed a significant increase in T888 phosphorylation of the high mannose 140 kDa form but not the 160 kDa complex carbohydrate form and there was no significant difference in the phosphorylation state of T888 in lysates prepared from cells exposed to either 3.0mM or 5.0mM Ca^{2+}_{o} (Fig. 6A and 6B). These results demonstrate that elevated Ca^{2+}_{o} stimulates CaSR-mediated PKC-dependent T888 phosphorylation of the predominantly intracellular 140 kDa form and the results above with the PKC inhibitor GF109203X indicate that T888 phosphorylation is required for the inhibitory effect of high Ca^{2+}_{o} on CYP27B1-dependent firefly luciferase protein expression and enzyme activity. Surprisingly, however, we did not observe enhanced T888 phosphorylation at a Ca^{2+}_{o} concentration of 5.0mM when compared with Ca^{2+}_{o} 3.0mM, suggesting that high Ca^{2+}_{o} lowers firefly luciferase expression via an additional molecular step.

Effect of $ERK_{1/2}$ Inhibitor Alone and in Combination With the PKC Inhibitor on CaSR-Mediated Control of Luciferase Activity

The roles of mitogen-activated protein kinase kinase (MEK) and $ERK_{1/2}$ in CaSR-mediated control of luciferase activity were assessed using PD184352 (10µM), a more potent and selective inhibitor of MEK than the previously used compound PD98059 [63, 64]. PD184352 appeared to have a marginal inhibitory effect on 3.0mM Ca^{2+}_{o}-stimulated RLA. In addition, PD184352, like the PKC inhibitor GF109203X, attenuated the inhibitory response at Ca^{2+}_{o} levels ≥ 5.0mM (Fig. 7A).

We then combined GF109203X (1µM) and PD184352 (10µM) to inhibit both PKC and MEK. Interestingly, this combination abrogated Ca^{2+}_{o}-dependent stimulation at both 3.0mM and 5.0mM Ca^{2+}_{o} Fig. 7B), demonstrating that Ca^{2+}_{o}-dependent stimulation can be supported by either CaSR-mediated stimulation of $ERK_{1/2}$ alone or PKC alone but cannot be supported by the CaSR if both pathways are blocked.

Role of Response Elements in the CYP27B1 Promoter

Finally, we investigated the role of the CYP27B1 promoter in luciferase expression, concentrating on the stimulatory effect at 3.0mM Ca^{2+}_{o}. To investigate the possible roles of response elements within the –1501 bp CYP27B1 promoter in CaSR-mediated increases in RLA and 1αOHase mRNA levels, the following truncated promoters, –305 bp, –200 bp, and –102 bp were studied (Table 1). The –305 bp promoter

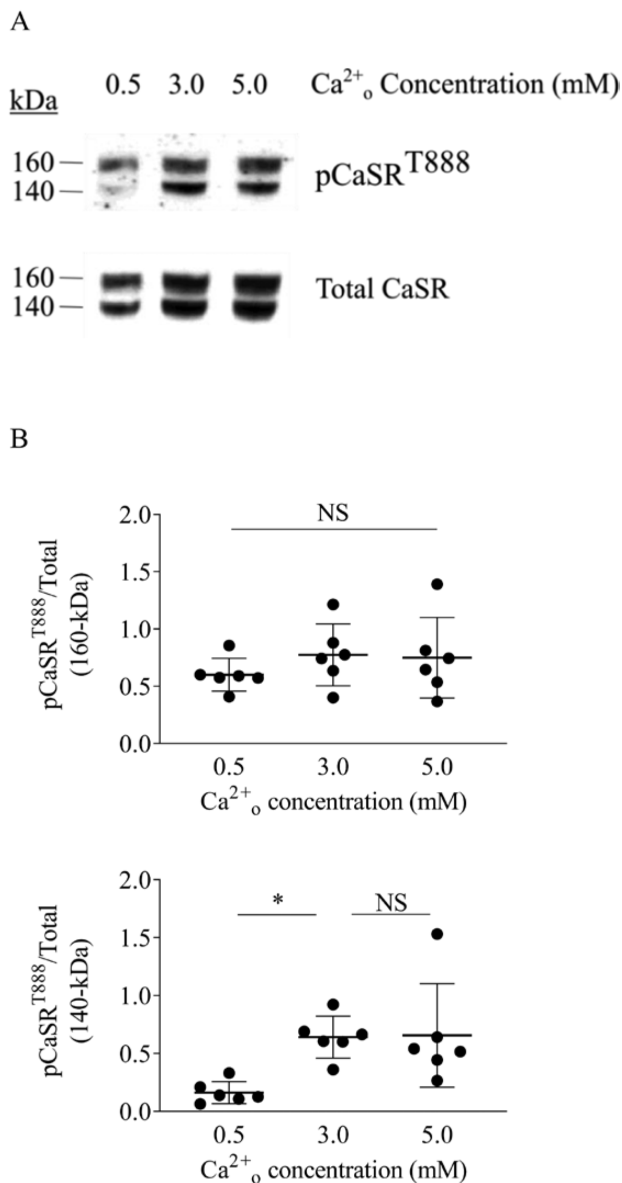

Figure 6. Impact of Ca^{2+}_o concentration on phosphorylation of CaSR residue T888. (A) Detection and quantitation of CaSR T888 phosphorylation, and total CaSR in HEK-CaSR cells that were incubated in 0.2% BSA supplemented DMEM at 0.5, 3.0, or 5.0mM Ca^{2+}_o for 24 hours, followed by Western blotting. Over the extended period of the experiment, we did not observe high Ca^{2+}_o-induced dephosphorylation of CaSR-T888. Representative Western blot from 6 independent experiments. (B) Quantitation of Western blot intensity data. In response to either 3.0mM or 5.0mM Ca^{2+}_o a stable increase in $pCaSR^{T888}$ was observed for the 140 kDa (high mannose) form but not the 160 kDa (complex glycosylated) form.

segment contains a number of putative response elements, including AP-1, NFkB, PU Box, CAAT Box(d), AP-2, CRE(p), Sp1(d), EBS(p), CCAAT, and Sp1(p) (Fig. 8A).

Basal CYP27B1 promoter–dependent luciferase activity, observed at 0.5mM Ca^{2+}_o, was unchanged following truncation to either −305 bp or −102 bp, but was significantly increased by 2- to 3-fold in lysates prepared from cells transfected with the -200 bp promoter ($P < 0.01$; Table 1). Upon exposure to 3.0mM Ca^{2+}_o, and irrespective of the basal level of promoter activity, we observed similar 2- to 2.5-fold increases in RLA in all cases, even in the −102 bp promoter ($P < 0.001$). These results demonstrate that Ca^{2+}_o-dependent increases in mRNA levels arose from response elements contained within the −102 bp basal promoter.

We thus focused on mutations of recognized response elements in the −102 bp promoter segment (CCAAT and Sp1(p)) in both the −102 bp and −305 bp promoter constructs. In the case of the CCAAT mutant but not the Sp1(p) mutant, we observed a striking loss of transcription and no Ca^{2+}_o-dependent stimulation. This finding indicates that CCAAT is absolutely required for transcription from the CYP27B1 promoter as described previously [22, 65] and suggests that it is also required for the response to high Ca^{2+}_o (Fig. 8B). If CCAAT is responsible for Ca^{2+}_o-dependent stimulation, as well as basal promoter activity, the fact that it has been reported previously to be activated by both PKC and $ERK_{1/2}$ would appear to be important [65]. Mutations of none of the other response elements tested, including Sp1(p), selectively disabled Ca^{2+}_o sensitivity (Supplemental Fig. 1; [48]).

Discussion

Previously, extracellular Ca^{2+} has been reported to control 1-alpha hydroxylase activity and thus the rate of 1,25-dihydroxyvitamin D synthesis both indirectly via, for example, impacts on PTH synthesis and secretion, and also directly in various tissue sites including the proximal tubule [27, 28], parathyroid [66], and osteoblasts [33]. However, the mechanisms by which Ca^{2+}_o modulates 1-alpha hydroxylase activity in these different tissue sites and acts to promote activity in some sites, such as parathyroid [66] and osteoblasts [33], but inhibits it in others, such as proximal tubule [27, 28], has not been determined. In considering how to investigate the molecular basis of these effects we noted that the CaSR, a key Ca^{2+}_o sensor with the ability to activate multiple signaling pathways downstream of the G proteins $G_{q/11}$, $G_{i/o}$, G_s, and $G_{12/13}$ [67], is expressed and functional in all these tissue sites (review: [30]).

Thus, in the present study we decided to test whether the CaSR is able to mediate Ca^{2+}_o-dependent control of CYP27B1 (1-alpha hydroxylase) expression using a well-defined model cell system, HEK-CaSR cells that were transfected with plasmid DNA encoding the CYP27B1 promoter upstream of firefly luciferase. In particular, we quantified CYP27B1-dependent changes in firefly luciferase activity in response to various concentrations of Ca^{2+}_o in the absence or presence of positive or negative modulators of the CaSR. HEK-CaSR cells, HEK-293

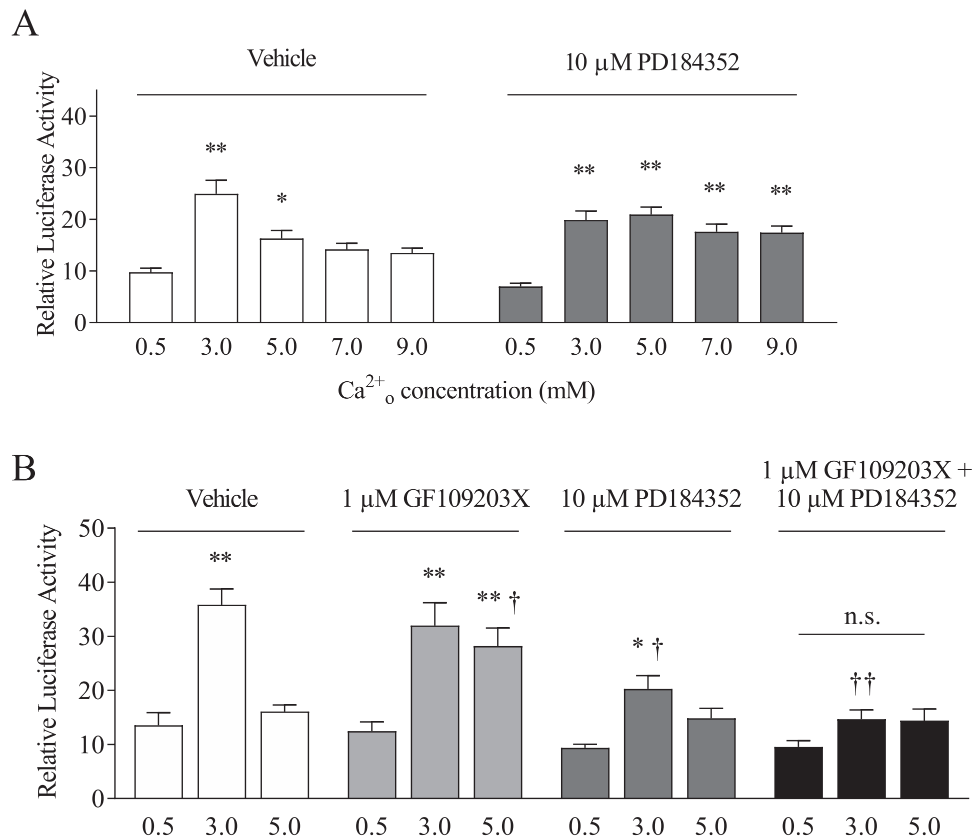

Figure 7. Effect of ERK inhibition and combined PKC/ERK inhibition on biphasic Ca^{2+}_{o} concentration–dependent control of CYP27B1-firefly luciferase. (A) Effect of MEK inhibitor, PD184352, on Ca^{2+}_{o}-dependent CYP27B1 −1501 bp promoter–controlled firefly luciferase expression in transfected HEK-CaSR cells. Luciferase assays were performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase reporter gene (CYP27B1 −1501 bp promoter), and the *Renilla* luciferase gene. Cells were preincubated 1 hour in 0.2% BSA supplemented DMEM at 0.5mM Ca^{2+}_{o} in the absence or presence of PD184352 (10μM). The Ca^{2+}_{o} concentration was then either maintained at 0.5mM or increased up to various concentrations (from 3.0mM to 9.0mM) for 24 hours. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.01$; ** $P < 0.001$ compared with 0.5mM Ca^{2+}_{o}. The data were obtained in 5 independent experiments, performed in triplicate. (B) Effects of PKC and MEK inhibitors alone and in combination on Ca^{2+}_{o}-dependent CYP27B1 promoter–dependent luciferase activity. Luciferase assays were performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase reporter gene (CYP27B1 −1501 bp promoter), and the *Renilla* luciferase gene. Cells were preincubated 1 hour in 0.2% BSA supplemented DMEM at 0.5mM Ca^{2+}_{o} in the presence of vehicle (0.2% DMSO), or GF109203X (1μM) or PD184352 (10μM), or both these compounds. The Ca^{2+}_{o} concentration was then either maintained at 0.5mM or increased to 3.0mM or 5.0mM for 24 hours. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.01$; ** $P < 0.001$ compared with 0.5mM Ca^{2+}_{o}; † $P < 0.01$; †† $P < 0.001$ compared with vehicle (0.2% DMSO). The data were obtained in 4 independent experiments performed in triplicate.

Table 1. Effect of truncating the CYP27B1 −1501 bp promoter to −305 bp, −200 bp, or −102 bp on Ca^{2+}_{o}-stimulated firefly luciferase expression in transfected HEK-CaSR cells

Promoter construct	Relative luciferase activity (RLA)		RLA (fold change)	*n*
	0.5mM Ca^{2+}_{o}	3.0mM Ca^{2+}_{o}	RLA (3mM Ca^{2+})/RLA (0.5mM Ca^{2+})	
−1501 bp	8.2 ± 0.6	21.2 ± 1.8	2.6 ± 0.3	10
−305 bp	6.4 ± 0.7	15.6 ± 1.8	2.4 ± 0.4	10
−200 bp	19.3 ± 3.6*	34.5 ± 5.4**	1.8 ± 0.4	5
−102 bp	5.3 ± 1.0	10.4 ± 2.1**	1.9 ± 0.5	10

Luciferase assays were performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase gene under the −1501 bp, −305 bp, −200 bp, or −102 bp CYP27B1 promoter, and the control *Renilla* luciferase gene under an HSV-TK promoter. Cells were incubated for 24 hours in 0.2% BSA supplemented DMEM at 0.5mM and 3.0mM Ca^{2+}_{o}. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.01$; ** $P < 0.001$ compared with −1501 bp.

Abbreviations: bp, base pair; RLA, Relative Luciferase Activity.

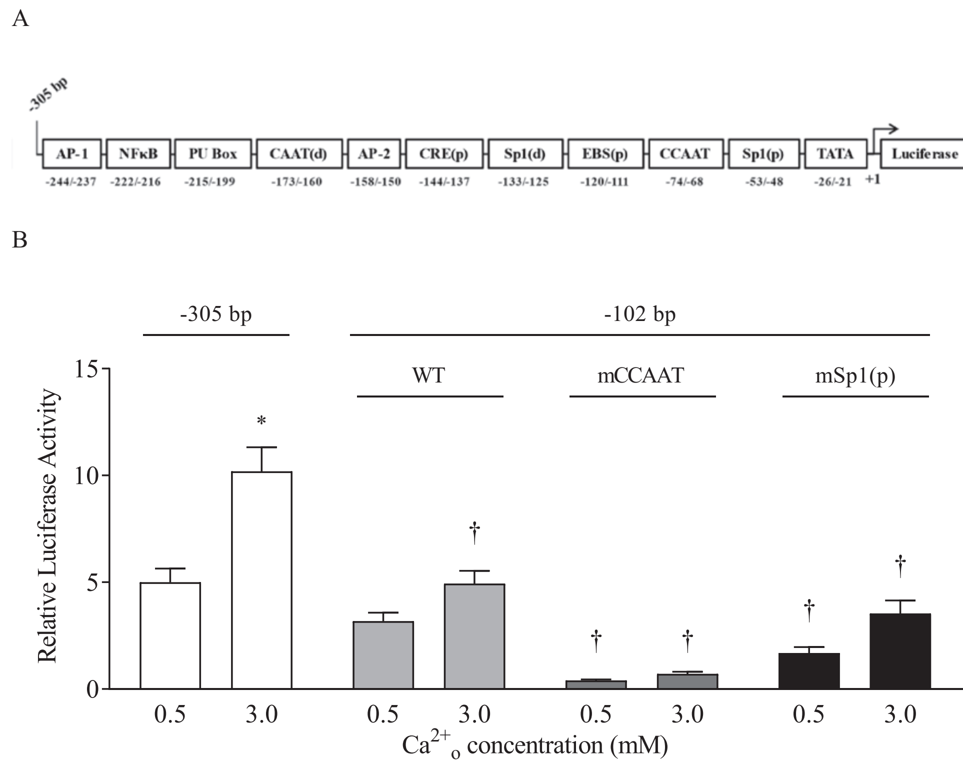

Figure 8. Impacts of selected mutations of response elements in the −102 bp CYP27B1 promoter on Ca^{2+}_o-dependent stimulation of CYP27B1-dependent firefly luciferase expression in transfected HEK-CaSR cells. (A) Schematic representation of the −305 bp promoter and putative response elements. Transcription factor binding sites and locations are shown as described in (21, 22, 86). Abbreviations: AP-1, activator protein 1 binding site; AP-2, activator protein 2 binding site; CRE, cAMP response element; EBS, Ets-binding site; NFκB, nuclear factor kappa light-chain enhancer binding site; PU-box, purine rich enhancer sequence; and Sp1, specificity protein 1 binding sites. (B) Luciferase assays were performed on HEK-CaSR cells transiently cotransfected with the firefly luciferase reporter gene under the −305 bp wild-type, −102 bp wild-type, −102 bp CCAAT mutated, or −102 bp Sp1(p) mutated CYP27B1 promoter, and the *Renilla* luciferase control gene under the HSV-TK promoter. Cells were incubated for 24 hours in 0.2% BSA supplemented DMEM at 0.5mM and 3.0mM Ca^{2+}_o. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. * $P < 0.001$ compared with 0.5mM Ca^{2+}_o; † $P < 0.001$ compared with −305 bp promoter. The data were obtained in 6 independent experiments performed in triplicate.

cells that stably express the CaSR, are a widely recognized model for studies of CaSR-mediated cell signaling responses [68-74], in which Ca^{2+}_o concentration–response relationships, such as those for Ca^{2+}_i mobilization [68] and PI-PLC [69, 70] are submaximal in the range 0.5mM to 3.0mM and maximal in the range 5.0mM to 8.0mM. In HEK-CaSR cells we demonstrated that a submaximal increase in Ca^{2+}_o to 3.0mM stimulated firefly luciferase activity 2- to 3-fold associated with increased levels of firefly luciferase mRNA and protein. Surprisingly, however, at a near-maximal concentration of Ca^{2+}_o (5.0mM) with respect to PI-PLC and Ca^{2+}_i mobilization, and at higher Ca^{2+}_o concentrations, we observed markedly reduced levels of firefly luciferase activity (Fig. 1A and 2A) and protein (Fig. 3C), but not mRNA (Fig. 4A), which was elevated to the same level observed at 3.0mM. Thus, the positive effect on luciferase activity that was observed as Ca^{2+}_o increased from 0.5mM to 3.0mM was dependent on CYP27B1-mediated transcription. However, the inhibitory mechanism that we observed as Ca^{2+}_o levels increased above 3.0mM occurred downstream of mRNA levels, either at the level of protein synthesis or via a posttranslational effect.

Neither phase of the Ca^{2+}_o-dependent response was observed in control HEK-293 cells transfected with the luciferase reporter genes (Fig. 2C). In addition, in luciferase-transfected HEK-CaSR cells, the Ca^{2+}_o-dependent biphasic response was: (i) shifted to the left by the CaSR PAM cinacalcet (1µM); and (ii) shifted to the right by the CaSR NAM NPS-2143 (1µM; Fig. 3A and 3B). Together these results demonstrate that the CaSR mediates both phases of the response, that is, both the positive transcriptional mechanism and the negative posttranscriptional mechanism. Increasing Ca^{2+}_o concentrations also increased endogenous 1αOHase mRNA levels in HEK-CaSR cells, and as in the case of luciferase mRNA under the CYP27B1 promoter, there was no evidence of suppression of 1αOHase mRNA levels at higher Ca^{2+}_o concentrations. Thus, at submaximal concentrations, the Ca^{2+}_o-stimulated-CaSR mediated activation of the CYP27B1 promoter and consequent transcriptional upregulation. This is in clear distinction to the effect of FGF23, which suppressed CYP27B1-dependent

1αOHase in alpha-klotho-transfected HEK-293 cells [19], consistent with its effect in the kidney (review: [75]).

To determine the mechanism that underlies Ca^{2+}_o-stimulated activation of firefly luciferase mRNA expression, we performed various truncations and point mutations of the CYP27B1 promoter. Ca^{2+}_o concentration–dependent stimulation of luciferase activity was maintained following truncation of the CYP27B1 promoter from −1501 bp to −305 bp from the transcriptional start site, and indeed to −200 bp and −102 bp. However, we observed significant changes in baseline luciferase activity with these truncations (enhanced with the truncation to −200 bp and reduced with the truncation to −102 bp). These findings demonstrate that Ca^{2+}_o sensitivity is closely linked to the transcriptional start site (Supplemental Fig. 1; Panel J; [48]). The −102 bp segment of the promoter contains 2 well-defined response elements, CCAAT (−74 bp to −68 bp) and Sp1(p) (−53 bp to −48 bp) ([22]; Fig. 8A). Of these, the CCAAT box (ATTGGCT) is critical for CYP27B1 promoter function and responds to the transcription factor C/EBPβ (review: [65]), which is activated by Ca^{2+}_i, PKC, and $ERK_{1/2}$ [76] (review: [65]). Mutation of the CCAAT box in the present study nearly abolished promoter function, as revealed by luciferase activity consistent with previous reports [22] and no longer supported Ca^{2+}_o-stimulated luciferase activity (Fig. 8B; Supplemental Fig. 1; [48]). Previously CCAAT box mutants were reported to abolish PTH-induced upregulation of CYP27B1 transcription although the proposed signaling mechanism required PKA [22]. In our studies mutation of the Sp1(p) site, on the other hand, suppressed total luciferase activity by around 50% without disturbing Ca^{2+}_o sensitivity. Thus, the results suggest that Ca^{2+}_o sensitivity is closely linked to the transcriptional start site of the CYP27B1 promoter and depends, at least in part, on the key CCAAT box response element at −74 bp to −68 bp downstream of the transcription factor C/EBPβ, which is activated by the 2 key cell signaling regulators we identified, PKC and $ERK_{1/2}$.

In the present study, the importance of PKC for CaSR-mediated control of CYP27B1-dependent firefly luciferase was demonstrated by the impact of the potent PKC inhibitor GF109203X, which converted the biphasic Ca^{2+}_o concentration–dependent response to a monophasic increase (Fig. 5A). As PKCα phosphorylates CaSR residue T888 in response to elevated Ca^{2+}_o and thereby modulates CaSR-dependent signaling [77], we hypothesized and demonstrated that T888 phosphorylation is required for high Ca^{2+}_o-dependent inhibition of CYP27B1-dependent firefly luciferase. We therefore tested the effect of various Ca^{2+}_o concentrations on luciferase activity in HEK-293 cells that stably expressed the nonphosphorylatable CaSR mutant T888A (HEK-$CaSR^{T888A}$ cells). Elevated Ca^{2+}_o (0.5mM to 3.0mM) stimulated luciferase activity in HEK-$CaSR^{T888A}$ cells but no secondary Ca^{2+}_o-mediated suppression of luciferase activity was observed at Ca^{2+}_o levels ≥ 5.0mM, even up to 12mM (Fig. 5B). Taken together, the results indicate that Ca^{2+}_o-dependent suppression of luciferase activity in HEK-CaSR cells is mediated by PKC-dependent phosphorylation of CaSR residue T888.

Over short periods (up to 30 minutes) as Ca^{2+}_o increases and the CaSR is activated in HEK-CaSR cells, $CaSR^{T888}$ is subjected first to phosphorylation and then to dephosphorylation as revealed by a $CaSR^{T888}$ phosphospecific antibody [57, 72]. We wondered whether a similar pattern of behavior might occur over the longer periods (2-24 hours) of the experiments described here. Interestingly, although we were readily able to detect T888 phosphorylation in response to moderately elevated Ca^{2+}_o, 3.0mM, after 24 hours (Fig. 6), exposure of the cells to a higher Ca^{2+}_o concentration, 5.0mM, did not suppress T888 phosphorylation toward the baseline level as expected (Fig. 6). These results appear to demonstrate that in response to sustained exposure to high Ca^{2+}_o concentrations, and following T888 dephosphorylation, which supports large stepwise increases in Ca^{2+}_i [72], a further phase of phosphorylation occurs that is dependent, at least in part, on PKC activity. Calyculin A and endothall inhibit T888 dephosphorylation and lower Ca^{2+}_i toward baseline in HEK-CaSR cells acutely exposed to elevated Ca^{2+}_o [72]. However, in preliminary experiments neither of these inhibitors altered either phase of the biphasic Ca^{2+}_o concentration–dependent firefly luciferase response. Thus, the results demonstrate that T888 phosphorylation, rather than dephosphorylation, is responsible for high Ca^{2+}_o-induced suppression of CYP27B1-dependent luciferase protein levels downstream of elevated luciferase mRNA levels. The results also suggest that the T888-phosphorylated CaSR activates a biochemical pathway that suppresses protein synthesis and/or promotes protein breakdown.

We also considered whether another well-known mediator of CaSR signaling, the protein kinase $ERK_{1/2}$ (review: [67]), might contribute to CaSR-mediated control of luciferase expression. $ERK_{1/2}$ is activated via several mechanisms downstream of the CaSR in HEK-293 cells, including $G_{q/11}$ [58], $G_{i/o}$ [78], transactivation of the EGF receptor [79], and β2 arrestin [80]. Interestingly, the activation of β2 arrestin is known to depend upon its binding to phosphorylated receptors (review: [81]). Thus, we tested the effect of PD184352 (10μM), a potent and selective inhibitor of MEK, which lies immediately upstream of $ERK_{1/2}$ in signaling pathways. PD184352 induced a right-shift in the Ca^{2+}_o concentration–response relationship such that the maximum response was observed at 5.0mM Ca^{2+}_o rather than at 3.0mM Ca^{2+}_o. In addition, it reduced the level

of secondary inhibition (with respect to baseline) from around 75% to 25% (Fig. 7B). These findings support the conclusion that $ERK_{1/2}$ mediates the inhibitory mechanism, presumably downstream of $CaSR^{T888}$ phosphorylation.

Surprisingly, when the PKC and MEK inhibitors were combined, $Ca^{2+}{}_o$-stimulated CYP27B1-luciferase was abolished (Fig. 7B). The results suggest that $Ca^{2+}{}_o$-stimulated CYP27B1-luciferase expression is supported: (i) in the presence of the PKC inhibitor GF109203X, by $ERK_{1/2}$; and (ii) in the presence of the MEK inhibitor PD184352, by PKC. Thus, CYP27B1 promoter–dependent transcription appears to be activated in parallel by PKC and $ERK_{1/2}$ converging on a common target sequence in the CCAAT box immediately upstream of the transcriptional start site.

The biphasic $Ca^{2+}{}_o$ concentration–response relationship we observed would appear to have physiological significance from 2 perspectives. Firstly, the inhibitory effect of $Ca^{2+}{}_o$ between submaximal and maximal concentrations would appear to be relevant to the control of renal proximal tubule 1αOHase activity, which is under tonic stimulation from PTH [20, 21]. If this is true, it provides further evidence for CaSR-mediated suppression of PTH-stimulated signaling in the proximal tubule. Previously, the CaSR was reported to disinhibit the PTH-induced inhibition of inorganic phosphate reabsorption [35]. Secondly, the stimulatory effect of $Ca^{2+}{}_o$ at submaximal concentrations would appear to be relevant to the control of 1αOHase activity in the parathyroid [32] and other extrarenal tissues including bone-forming osteoblasts [33].

Mechanism Underlying $Ca^{2+}{}_o$-Dependent Inhibition of 1-Alpha Hydroxylase

In the present study, while we identified a transcriptional mechanism for the upregulation of CYP27B1 mRNA at submaximal $Ca^{2+}{}_o$, at maximal $Ca^{2+}{}_o$ concentrations we observed reductions in luciferase activity and protein levels that were not accompanied by a reduction in mRNA levels. This finding is similar to that reported for 1αOHase in renal proximal tubular cells [28], raising the possibility that a common inhibitory mechanism that is $Ca^{2+}{}_o$-stimulated and CaSR-mediated may operate in both cases. If this is true, it is not transcriptional and it does not arise from impaired mRNA stability as firefly luciferase mRNA levels remained stably elevated over the 24-hour time course of these experiments at all $Ca^{2+}{}_o$ concentrations tested above 3.0mM. For this reason, it seems likely that the inhibitory effect was exerted on translational or posttranslational activity, such as via enhanced protein breakdown.

While the effect might be exerted by a negative effect on translation, a mechanism of this type seems unlikely for two reasons. Firstly, although we identified a target sequence in the native 3′-UTR of *P. pyralis* luciferase, for a miRNA, miR-374, whose levels are modulated by the CaSR [82], in the plasmid construct we used, the native luciferase 3′UTR was replaced with a shortened 31-base sequence, which does not contain any recognized miR target sequences (mirdb.org). Secondly, although cell signaling pathways can control general rates of translation, such as via the regulation of mTOR and S6 kinase activity, we did not observe any $Ca^{2+}{}_o$-stimulated suppression of *Renilla* luciferase activity in experiments in which high $Ca^{2+}{}_o$ robustly lowered firefly luciferase activity and protein levels (Figs. 1-3).

Thus, it seems more likely that the inhibitory effect is exerted via a signaling pathway linked to a posttranslational effect, such as protein breakdown. In our Western blot studies of $Ca^{2+}{}_o$-stimulated control of luciferase protein expression we did not observe lower molecular weight fragments that might have pointed to a proteolytic mechanism. On the other hand, $Ca^{2+}{}_i$-dependent and -independent signaling pathways have been identified that stimulate the intracellular activation and/or release of proteases. The cell-permeant inhibitor of the $Ca^{2+}{}_i$-dependent protease calpain, Calpain Inhibitor I, did not prevent high $Ca^{2+}{}_o$-induced suppression of luciferase activity in the present study. Another candidate is the mitochondrial serine protease Omi/HtrA, which localizes to the mitochondrial intermembrane space in close proximity to 1α-OHase. Omi/HtrA was previously shown to be activated and released via a PP2A-dependent signaling pathway [83]. The $Ca^{2+}{}_o$-stimulated CaSR activates PP2A in association with T888 phosphorylation [72] and may thus induce the activation and release of Omi/HtrA with the ability to cleave luciferase and perhaps other cellular protein targets. Consistent with this idea, firefly luciferase is highly sensitive to proteolytic degradation [84], and a specific serine protease-selective site has been identified [85]. 1αOHase is also highly sensitive to proteolytic degradation by proteases, including the serine proteases trypsin and chymotrypsin but not caspases (https://web.expasy.org/peptide_cutter/) and PP2A-stimulated activation and/or release of the serine protease Omi/HtrA might thereby provide a mechanism by which maximal $Ca^{2+}{}_o$ stimulation of the CaSR suppresses 1αOHase activity. Based on our findings, the possibility that the CaSR mediates proteolytic degradation of 1αOHase requires further consideration.

Finally, it might be wondered what impacts activating and inactivating CaSR mutations linked to human autosomal dominant hypocalcemia and familial hypocalciuric hypercalcemia [30] respectively might have on 1αOHase activity. In fact, it seems plausible that the effects may be minor due to the compensatory effect of a reduced plasma

calcium concentration in the context of activating mutations and an increased plasma calcium concentration in the context of inactivating mutations.

In conclusion, we have demonstrated CaSR-mediated biphasic Ca^{2+}_o concentration–dependent control of CYP27B1 promoter–dependent firefly luciferase activity in HEK-293 cells. At *submaximal* Ca^{2+}_o, the CaSR mediates Ca^{2+}_o-stimulated activation of the CYP27B1 promoter dependent on 2 parallel signaling pathways in which the CaSR links to PKC and $ERK_{1/2}$ and converges on the CCAAT box immediately upstream of the transcriptional start site. At *maximal* Ca^{2+}_o, the CaSR mediates a posttranscriptional mechanism, most probably protein breakdown, dependent upon a mechanism that links the CaSR to phosphorylation of CaSR residue T888 via PKCα and to an $ERK_{1/2}$-dependent pathway, presumably downstream of $CaSR^{T888}$ phosphorylation. The results demonstrate the existence of complex Ca^{2+}_o-dependent control of CYP27B1 promoter–dependent firefly luciferase and are consistent with hypotheses that the CaSR mediates both positive control of 1αOHase activity in the parathyroid, skeleton, and other extrarenal tissues, along with negative control of 1αOHase activity in the renal proximal tubule.

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