Calcium-sensing receptors control CYP27B1-luciferase expression. Transcriptional and post-transcriptional mechanisms.

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Abstract

25-hydroxyvitamin D 1α-hydroxylase (encoded by CYP27B1), which catalyses the synthesis of 1,25-dihydroxyvitamin D₃, is subject to negative or positive modulation by extracellular Ca²⁺ (Ca²⁺o) depending on the tissue. However, the Ca²⁺ sensors and underlying mechanisms are unidentified. We tested whether calcium-sensing receptors (CaSRs) mediate Ca²⁺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, co-transfected with reporter genes for CYP27B1-Photinus pyralis (firefly) luciferase and control Renilla luciferase, an increase in Ca²⁺o from 0.5 to 3.0 mM induced a 2-3 fold increase in firefly-luciferase activity as well as mRNA and protein levels. Surprisingly, firefly-luciferase was specifically suppressed at Ca²⁺o ≥ 5.0 mM, demonstrating biphasic Ca²⁺o control. Both phases were mediated by CaSRs as revealed by positive and negative modulators. However, Ca²⁺o induced simple monotonic increases in firefly-luciferase and endogenous CYP27B1 mRNA levels, indicating that the inhibitory effect of high Ca²⁺o was post-transcriptional. Studies with inhibitors and the CaSR C-terminal mutant T888A identified roles for PKC, phosphorylation of T888, and ERK₁/₂ in high Ca²⁺o-dependent suppression of firefly-luciferase. Blockade of both PKC and ERK₁/₂ abolished Ca²⁺o-stimulated firefly-luciferase, demonstrating that either PKC or ERK₁/₂ is sufficient to stimulate the CYP27B1 promoter. A key CCAAT box (−74 bp to −68 bp), which is regulated downstream of PKC and ERK₁/₂ was required for both basal transcription and Ca²⁺o-mediated transcriptional upregulation. The CaSR mediates Ca²⁺o-dependent transcriptional upregulation of 1α-hydroxylase and an additional CaSR-mediated mechanism is identified by which Ca²⁺o can promote luciferase and possibly 1α-hydroxylase breakdown.

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

The mitochondrial membrane enzyme 25-hydroxyvitamin D 1α-hydroxylase (1αOHase), which is encoded by the CYP27B1 gene, catalyses the synthesis of 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃; calcitriol). The hormonal form of 1,25(OH)₂D₃, 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 ionised calcium and phosphate concentrations, and mineralisation 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)₂D₃ generated locally in extra-renal tissues via 1αOHase encoded by the same CYP27B1 gene, acts via VDR-dependent and VDR-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 bacteriocidal 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)₂D₃ (15), and FGF-23 (16-19), which are inhibitory. These hormones act, at least in part, on the promoter region of the CYP27B1 gene via receptor-dependent signalling mechanisms. Thus, PTH (20-23) and calcitonin (24) increase and 1,25(OH)₂D₃ (21,25) and FGF-23 (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$^{2+}$ (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 i.e., directly on cells of the proximal tubule, parathyroid, and osteoblast lineage. Thus Ca$^{2+}$, 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$^{2+}$ on 1αOHase activity in the proximal tubule and stimulatory effect of Ca$^{2+}$ on 1αOHase activity in extra-renal 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$^{2+}$-dependent effects. In the parathyroid the CaSR mediates Ca$^{2+}$-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$^{2+}$-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 mineralisation ((36); review: (37)) at least in part via a newly described signalling 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$^{2+}$ on 1,25(OH)$_2$D$_3$ synthesis via 1αOHase, including suppressed synthesis in the renal proximal tubule and/or
stimulated synthesis in extra-renal 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 exhibit downregulation of the CaSR (e.g., (44)) and other key receptors (e.g., 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\textsuperscript{2+}-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 two immediate early genes, Egr-1 and AP-1 (46). To facilitate our analyses, HEK-CaSR cells were transfected with a CYP27B1 promoter-\textit{Photinus pyralis} firefly luciferase reporter gene and a control, Herpes Simplex Virus (HSV) thymidine kinase (TK) promoter-\textit{Renilla} luciferase construct as described previously (47).

To our surprise, we found that the CaSR mediates a complex, biphasic Ca\textsuperscript{2+} concentration-dependent response. At submaximal Ca\textsuperscript{2+} concentrations (0.5–3.0 mM), the CaSR mediated transcriptional upregulation of CYP27B1-firefly luciferase. However, at maximal Ca\textsuperscript{2+} concentrations (≥ 5.0 mM), the CaSR mediated a post-transcriptional down-regulation of luciferase protein levels. Although luciferase and 1αOHase are clearly distinct proteins, the results indicate that at high Ca\textsuperscript{2+} 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 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% CO₂ 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 h 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 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 25-30 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 Suppl. 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 Ascl restriction site included on the 5’ ends. The PCR product was digested with 5 U DpnI and 5 U Ascl, 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 Suppl. Table 2 (48).

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

**Transfection of HEK-CaSR cells with plasmid DNA**

HEK-CaSR cells plated into 48-well plates were incubated in Ca²⁺-free DMEM (Gibco) supplemented with 0.5 mM Ca²⁺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 h.

**Cell incubations**

Cells were incubated for 24 h in Ca²⁺-free DMEM (Gibco 21068) supplemented with 0.2% bovine serum albumin (BSA; Sigma Aldrich A9576) and adjusted for Ca²⁺o using CaCl₂. 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 signalling pathways, inhibitors were used to pretreat cells for 1 h prior to 24 h incubation. Following treatment, the cells were washed once in chilled phosphate-buffered saline (PBS; 137 mM NaCl, 2.7
mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and lysates prepared as required for Dual-Luciferase® Reporter (DLR™) Assay System (Promega E1910), quantitative real-time 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 according to the manufacturer’s instructions. Bioluminescence measurements were normalised for background (untransfected cell lysates), and expressed either as raw bioluminescence counts or as Relative Luciferase Activities i.e., 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 PCRs 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-HRP conjugated (goat polyclonal, Abcam #97051; (52)); Anti-CaSR phosphoT888 (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 (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1x Protease Inhibitor Cocktail (Cell Signaling Technology)) and triturated 30 times through a 21 G needle. In some experiments the RIPA-like lysis buffer contained 12 mM HEPES (pH 7.6), 300 mM mannitol, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton-X 100, 0.1% (w/v) SDS, 1 mM NaF, 100 μM sodium vanadate, 250 μM sodium pyrophosphate, 1.25 μM pepstatin, 4 μM leupeptin and 4.8 μM 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 visualised 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 one-way analysis of variance (1-way ANOVA) for Ca\(^{2+}\) concentration-response experiments, and two-way analysis of variance (2-way ANOVA) for studies involving CaSR modulators and inhibitors of
signalling, 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\(^{2+}\) on luciferase activity*

HEK-CaSR cells or control HEK-293 cells were transiently co-transfected 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\(^{2+}\) concentrations to determine the impact of CaSR activation on firefly luciferase, as a measure of CYP27B1 promoter function, and control *Renilla* luciferase activity.

In three initial experiments we incubated transfected HEK-CaSR cells with three different Ca\(^{2+}\) concentrations, 0.5, 3.0 and 5.0 mM and analysed both firefly and *Renilla* luciferase activities at 2, 4, 8 and 24 h (Fig. 1). At 0.5 mM Ca\(^{2+}\), there was no change in the baseline level of either firefly or *Renilla* luciferase at any of the time-points over the 24 h period of the experiment. However, at 3.0 mM Ca\(^{2+}\) we observed a linear increase in firefly luciferase, but not *Renilla* luciferase, that was apparent after 4 h and reached a level of around 2-4 fold of baseline after 24 h (Fig. 1A). There was no significant change in *Renilla* luciferase activity from 2-24 h at 3.0 mM Ca\(^{2+}\). Interestingly, at 5.0 mM Ca\(^{2+}\) we observed no significant changes in either firefly or *Renilla* luciferase at any of the time-points studied over the course of the 24 h period. Thus, we observed a striking biphasic Ca\(^{2+}\) concentration response for firefly luciferase, but not *Renilla* luciferase, which was maximal at 3.0 mM Ca\(^{2+}\) (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* and selected 24 h as the standard incubation time.
We confirmed and extended these observations in subsequent experiments. Thus, HEK-CaSR cells elevated Ca\(^{2+}\) (3.0 mM) stimulated firefly luciferase activity by 3-4 fold over 24 h but had little or no effect on Renilla luciferase (Fig. 2A). However, as described above, HEK-CaSR cells exposed to 5.0 mM or 8.0 mM Ca\(^{2+}\) for 24 h exhibited markedly reduced firefly luciferase activity when compared to the peak response at 3.0 mM Ca\(^{2+}\) (p < 0.05), lying close to the baseline level observed at 0.5 mM Ca\(^{2+}\) (Fig. 2A). No effect, however, was observed on Renilla luciferase.

When the results in Fig. 2A were expressed as RLA, elevated Ca\(^{2+}\) (3.0 mM) induced a 2-3 fold increase over 24 h when compared to control Ca\(^{2+}\) (0.5 mM) 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 5.0 mM or 8.0 mM Ca\(^{2+}\) for 24 h exhibited markedly reduced RLA when compared to the peak response at 3.0 mM Ca\(^{2+}\) (p < 0.05). These findings indicate that an increase in Ca\(^{2+}\) from 0.5 mM to 3.0 mM activated a CaSR-dependent stimulatory signalling pathway upstream of firefly luciferase expression but that a further increase in Ca\(^{2+}\) to 5.0 mM or higher concentrations activated an inhibitory signalling pathway. Thus, the findings point to the existence of a biphasic response in the Ca\(^{2+}\) range 0.5-5.0 mM with a peak at approximately 3.0 mM. Neither the stimulatory nor the inhibitory phases of the Ca\(^{2+}\)-dependent biphasic response in RLA could be detected at shorter time points ≤ 4 h (three 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 3.0 mM Ca\(^{2+}\)-induced stimulation of firefly luciferase expression or 5.0 mM Ca\(^{2+}\)-induced suppression.
Cinacalcet (1 µM) markedly enhanced RLA in cells exposed to control Ca\(^{2+}\) (0.5 mM) for 24 h and also suppressed RLA at 3.0 mM (\(p < 0.01\); Fig. 3A). However, there was no apparent change at 5.0 mM (not shown). Thus, cinacalcet shifted the biphasic Ca\(^{2+}\) concentration-response relationship to the left, indicating that it had increased the Ca\(^{2+}\) 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\(^{2+}\)-concentration response curve. Thus, RLA was unchanged at the control Ca\(^{2+}\) of 0.5 mM but significantly suppressed at Ca\(^{2+}\) 3.0 mM (\(p < 0.01\); Fig. 3B) and further increases in Ca\(^{2+}\) to 5.0 or 7.0 mM stimulated, rather than inhibited, RLA by around 3-fold, compared to the 0.5 mM Ca\(^{2+}\) control (\(p < 0.001\) compared to 0.5 mM Ca\(^{2+}\)).

Similar results were obtained for firefly luciferase protein expression by western blotting, in which band intensities increased 2.9 ± 0.4-fold at 3.0 mM Ca\(^{2+}\) when compared to 0.5 mM Ca\(^{2+}\) then decreased at 5.0 mM Ca\(^{2+}\) 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\(^{2+}\) concentration-dependent changes in RLA arose from CaSR-mediated biphasic Ca\(^{2+}\) concentration-dependent changes in firefly luciferase protein levels.

_Elevated Ca\(^{2+}\) increases luciferase mRNA expression_

We next performed quantitative RT-PCR for luciferase mRNA to investigate whether either 3.0 mM Ca\(^{2+}\)-dependent stimulation or 5.0 mM Ca\(^{2+}\)-dependent suppression of firefly luciferase activity was dependent on changes in firefly luciferase mRNA levels, i.e., 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 2-24 h and selected 24 h 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\(^{2+}\) from 0.5 mM to 3.0 mM (p < 0.01; Fig. 4A), which resembled the observed increase in firefly luciferase enzyme activity (Figs. 1, 2). The results are consistent with the conclusion that in HEK-CaSR cells exposed to 3.0 mM Ca\(^{2+}\), 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 difference in the level of firefly luciferase mRNA between that observed at 3.0 mM Ca\(^{2+}\) and that observed at 5.0 mM Ca\(^{2+}\), which exhibited a 2.7 ± 0.6-fold elevation when compared to the 0.5 mM Ca\(^{2+}\) control. The results indicate that the inhibition of firefly luciferase activity observed at 5.0 mM Ca\(^{2+}\) when compared to 3.0 mM Ca\(^{2+}\) did not arise from Ca\(^{2+}\)-dependent inhibition of transcription or loss of mRNA stability.

**Ca\(^{2+}\)-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\(^{2+}\). 1αOHase mRNA levels exhibited an apparent 2-3 fold increase at 3.0 mM Ca\(^{2+}\) and a 4-6-fold increase at 5.0 mM Ca\(^{2+}\) at 24 h. We were unable to detect, however, any Ca\(^{2+}\)-dependent changes in 1αOHase protein, which was at the limit of sensitivity of the western blot assay.

We selected 24 h for subsequent experiments on the Ca\(^{2+}\) concentration dependence of 1αOHase mRNA expression over an extended Ca\(^{2+}\) range of 0.5-7.0 mM. Increasing Ca\(^{2+}\) from 0.5 mM to 3.0, 5.0, or 7.0 mM increased 1αOHase mRNA levels in HEK-CaSR cells, with a maximal 3.4 ± 0.4-fold...
stimulation observed at 5.0 mM Ca\textsuperscript{2+} (p < 0.001; Fig. 4B) and no significant difference in 1\alpha OHase mRNA expression was observed as Ca\textsuperscript{2+} was increased between 5.0 and 7.0 mM Ca\textsuperscript{2+}. Thus, we did not observe biphasic Ca\textsuperscript{2+} concentration-dependent control of 1\alpha OHase mRNA expression in HEK-CaSR cells.

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

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 1\alpha OHase mRNA levels in HEK-CaSR cells we investigated the impacts on CYP27B1 promoter-dependent luciferase protein levels of selective inhibitors of key signalling pathways downstream of the CaSR, focusing first on PKC. Several PKC isoforms, including α and ε, are activated in response to the Ca\textsuperscript{2+}-stimulated CaSR (58,59) and PKC is activated by either PI-PLC-dependent and/or -independent mechanisms downstream of GPCRs (review: (60)). To investigate the role of PKC, HEK-CaSR cells were preincubated for 1 h in the absence or presence of the potent PKC inhibitor GF109203X, a known inhibitor of PKC α, β\textsubscript{1}, β\textsubscript{2}, and γ (61) and the cells were then incubated for a further 24 h in the continuing presence of GF109203X at 0.5, 3.0, or 5.0 mM Ca\textsuperscript{2+}. GF109203X (0.5 and 1 µM) had no effect on basal RLA at 0.5 mM Ca\textsuperscript{2+} or on 3.0 mM Ca\textsuperscript{2+}-induced stimulation of RLA. Surprisingly, however, GF109203X (1 µM) reversed the inhibitory effect observed at 5.0 mM Ca\textsuperscript{2+} (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 $\mathrm{Ca}^{2+}$ concentration-dependent relationship in HEK-CaSR$^{T888A}$ cells. For this reason, we performed the experiments over an extended $\mathrm{Ca}^{2+}$ range (0.5-12 mM).

HEK-CaSR$^{T888A}$ cells exhibited $\mathrm{Ca}^{2+}$-stimulated firefly luciferase expression at 3.0 mM 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 0.5 mM $\mathrm{Ca}^{2+}$ in HEK-CaSR$^{T888A}$ cells ($p < 0.05$; Fig. 5B).

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

*Chronically elevated $\mathrm{Ca}^{2+}$ promotes CaSR T888 phosphorylation*

The effects of various $\mathrm{Ca}^{2+}$ 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 $\mathrm{Ca}^{2+}$ from 0.5 to 3.0 or 5.0 mM over 24 h, 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 3.0 mM or 5.0 mM $\mathrm{Ca}^{2+}$ (Fig. 6A, B). These results demonstrate that elevated $\mathrm{Ca}^{2+}$ 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\textsuperscript{2+} on CYP27B1-dependent firefly luciferase protein expression and enzyme activity. Surprisingly, however, we did not observe enhanced T888 phosphorylation at a Ca\textsuperscript{2+} concentration of 5.0 mM when compared to Ca\textsuperscript{2+} 3.0 mM suggesting that high Ca\textsuperscript{2+} lowers firefly luciferase expression via an additional molecular step.

**Effect of ERK\textsubscript{1/2} inhibitor alone and in combination with the PKC-inhibitor on CaSR-mediated control of luciferase activity**

The roles of MEK and ERK\textsubscript{1/2} in CaSR-mediated control of luciferase activity were assessed using PD184352 (10 μM), 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 3.0 mM Ca\textsuperscript{2+}-stimulated RLA. In addition, PD184352, like the PKC inhibitor GF109203X, attenuated the inhibitory response at Ca\textsuperscript{2+} levels ≥ 5.0 mM (Fig. 7A).

We then combined GF109203X (1 μM) and PD184352 (10 μM) to inhibit both PKC and MEK. Interestingly, this combination abrogated Ca\textsuperscript{2+}-dependent stimulation at both 3.0 and 5.0 mM Ca\textsuperscript{2+} (Fig. 7B) demonstrating that Ca\textsuperscript{2+}-dependent stimulation can be supported by either CaSR-mediated stimulation of ERK\textsubscript{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 3.0 mM Ca\textsuperscript{2+}. 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 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 0.5 mM Ca\textsuperscript{2+}, was unchanged following truncation to either −305 bp or −102 bp, but was significantly increased by 2-3 fold in lysates prepared from cells in which the −200 bp promoter was expressed (p < 0.01; Table 1). Upon exposure to 3.0 mM Ca\textsuperscript{2+}, and irrespective of the basal level of promoter activity, we observed similar 2-2.5-fold increases in RLA in all cases, even in the −102 bp promoter (p < 0.001). These results demonstrate that Ca\textsuperscript{2+}-dependent increases in mRNA levels arose from response elements contained within the −102 bp basal promoter.

We thus focused on mutations of recognised 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\textsuperscript{2+}-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\textsuperscript{2+} (Fig. 8B). If CCAAT is responsible for Ca\textsuperscript{2+}-dependent stimulation, as well as basal promoter activity, the fact that it has been reported previously to be activated by both PKC and ERK\textsubscript{1/2} would appear to be important (65). Mutations of none of the other response elements tested including Sp1(p) selectively disabled Ca\textsuperscript{2+}-sensitivity (Suppl. Fig. 1; (48)).

Discussion

Previously, extracellular Ca\textsuperscript{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\textsuperscript{2+} modulates 1-alpha
hydroxylase activity in these different tissue sites and acts to promote activity in some sites e.g., parathyroid (66), and osteoblasts (33); but inhibits it in others e.g., 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\textsuperscript{2+} sensor with the ability to activate multiple signaling pathways downstream of the G proteins G\textsubscript{q/11}, G\textsubscript{i/o}, G\textsubscript{s}, and G\textsubscript{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\textsuperscript{2+}-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\textsuperscript{2+} in the absence or presence of positive or negative modulators of the CaSR. HEK-CaSR cells, HEK-293 cells that stably express the CaSR, are a widely recognised model for studies of CaSR-mediated cell signalling responses (68-74), in which Ca\textsuperscript{2+} concentration-response relationships, such as those for Ca\textsuperscript{2+}i mobilisation (68) and PI-PLC (69,70) are submaximal in the range 0.5 - 3.0 mM and maximal in the range 5.0-8.0 mM. In HEK-CaSR cells we demonstrated that a submaximal increase in Ca\textsuperscript{2+} to 3.0 mM stimulated firefly luciferase activity 2-3 fold associated with increased levels of firefly luciferase mRNA and protein. Surprisingly, however, at a near-maximal concentration of Ca\textsuperscript{2+} (5.0 mM) with respect to PI-PLC and Ca\textsuperscript{2+}i mobilisation, and at higher Ca\textsuperscript{2+} concentrations, we observed markedly reduced levels of firefly luciferase activity (Fig. 1A, 2A) and protein (Fig. 3C), but not mRNA (Fig. 4A), which was elevated to the same level observed at 3.0 mM. Thus, the positive effect on luciferase activity that was observed as Ca\textsuperscript{2+} increased from 0.5 mM to 3.0 mM was dependent on CYP27B1-mediated transcription. However, the inhibitory mechanism that we observed as Ca\textsuperscript{2+} levels increased above 3.0 mM occurred downstream of mRNA levels, either at the level of protein synthesis or via a post-translational effect.
Neither phase of the Ca\textsuperscript{2+}-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\textsuperscript{2+}-dependent biphasic response was: (i) shifted to the left by the CaSR PAM, cinacalcet (1 µM); and (ii) shifted to the right by the CaSR NAM, NPS-2143 (1 µM; Fig. 3A and 3B). Together these results demonstrate that the CaSR mediates both phases of the response, i.e., both the positive transcriptional mechanism and the negative post-transcriptional mechanism. Increasing Ca\textsuperscript{2+} 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\textsuperscript{2+} concentrations. Thus at submaximal concentrations, the Ca\textsuperscript{2+}-stimulated-CaSR mediated activation of the CYP27B1 promoter and consequent transcriptional upregulation. This is in clear distinction to the effect of FGF-23, 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\textsuperscript{2+}-stimulated activation of firefly luciferase mRNA expression, we performed various truncations and point mutations of the CYP27B1 promoter. Ca\textsuperscript{2+} 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\textsuperscript{2+} sensitivity is closely linked to the transcriptional start site (Suppl. Fig. 1; Panel J; (48)). The –102 bp segment of the promoter contains two 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\textsuperscript{2+}, PKC, and ERK\textsubscript{1,2} in CaSR-expressing HEK-293 cells (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\textsuperscript{2+}-stimulated luciferase activity (Fig. 8B; Suppl. Fig. 1; (48)). Previously CCAAT box mutants were reported to abolish PTH-induced upregulation of CYP27B1 transcription although the proposed signalling 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\textsuperscript{2+} sensitivity. Thus the results suggest that Ca\textsuperscript{2+} 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/EBP\(\beta\), which is activated by the two key cell signalling 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\textsuperscript{2+} concentration dependent response to a monophasic increase (Fig. 5A). As PKC\(\alpha\) phosphorylates CaSR residue T888 in response to elevated Ca\textsuperscript{2+} and thereby modulates CaSR-dependent signalling (77), we hypothesised and demonstrated that T888 phosphorylation is required for high Ca\textsuperscript{2+}-dependent inhibition of CYP27B1-dependent firefly luciferase. We therefore tested the effect of various Ca\textsuperscript{2+} concentrations on luciferase activity in HEK-293 cells that stably expressed the non-phosphorylatable CaSR mutant T888A (HEK-CaSR\textsuperscript{T888A} cells). Elevated Ca\textsuperscript{2+} (0.5-3.0 mM) stimulated luciferase activity in HEK-CaSR\textsuperscript{T888A} cells but no secondary Ca\textsuperscript{2+}-mediated suppression of luciferase activity was observed at Ca\textsuperscript{2+} levels \(\geq 5.0\) mM, even up to 12 mM (Fig. 5B). Taken together, the results indicate that Ca\textsuperscript{2+}-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 min) as Ca\textsuperscript{2+} increases and the CaSR is activated in HEK-CaSR cells, CaSR\textsuperscript{T888} is subjected first to phosphorylation and then to dephosphorylation as revealed by a CaSR\textsuperscript{T888} phospho-specific antibody (57,72). We wondered whether a similar pattern of behavior
might occur over the longer periods (2-24 h) of the experiments described here. Interestingly, although we were readily able to detect T888 phosphorylation in response to moderately elevated Ca\(^{2+}\_o\) 3.0 mM after 24 h (Fig. 6), exposure of the cells to a higher Ca\(^{2+}\_o\) concentration, 5.0 mM did not suppress T888 phosphorylation towards 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 step-wise 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\(^{2+}\_i\) towards 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 signalling, 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 \(\beta_2\) arrestin (80). Interestingly, the activation of \(\beta_2\) arrestin is known to depend upon its binding to phosphorylated receptors (review: (81)). Thus, we tested the effect of PD184352 (10 µM), a potent and selective inhibitor of MEK, which lies immediately upstream of ERK\(_{1/2}\) in signalling pathways. PD184352 induced a right-shift in the Ca\(^{2+}\_o\) concentration-response relationship such that the maximum response was observed at 5.0 mM Ca\(^{2+}\_o\) rather than at 3.0 mM 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\textsubscript{1/2} mediates the inhibitory mechanism, presumably downstream of CaSR\textsuperscript{T888} phosphorylation.

Surprisingly, when the PKC and MEK inhibitors were combined, Ca\textsuperscript{2+}-stimulated CYP27B1-luciferase was abolished (Fig. 7B). The results suggest that Ca\textsuperscript{2+}-stimulated CYP27B1-luciferase expression is supported: (i) in the presence of the PKC inhibitor GF109203X, by ERK\textsubscript{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\textsubscript{1/2} converging on a common target sequence in the CCAAT box immediately upstream of the transcriptional start site.

The biphasic Ca\textsuperscript{2+} concentration-response relationship we observed would appear to have physiological significance from two perspectives. Firstly, the inhibitory effect of Ca\textsuperscript{2+} between submaximal and maximal concentrations would appear to be relevant to the control of renal proximal tubule 1\(\alpha\)OHase 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\textsuperscript{2+} at submaximal concentrations would appear to be relevant to the control of 1\(\alpha\)OHase activity in the parathyroid (32) and other extra-renal tissues including bone-forming osteoblasts (33).

**Mechanism underlying Ca\textsuperscript{2+}-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\textsuperscript{2+}, at maximal Ca\textsuperscript{2+} 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 1\(\alpha\)OHase in renal proximal tubular cells (28), raising the possibility that a common inhibitory mechanism that is Ca\textsuperscript{2+}-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 h time-course of these experiments at all Ca\textsuperscript{2+}i concentrations tested above 3.0 mM. For this reason, it seems likely that the inhibitory effect was exerted on translational or post-translational activity, such as via enhanced protein breakdown.

While the results 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 recognised miR target sequences (mirdb.org). Secondly, although cell signalling pathways can control general rates of translation, such as via the regulation of mTOR and S6 kinase activity, we did not observe any Ca\textsuperscript{2+}i-stimulated suppression of _Renilla_ luciferase activity in experiments in which high Ca\textsuperscript{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 signalling pathway linked to a post-translational effect, such as protein breakdown. In our western blot studies of Ca\textsuperscript{2+}i-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\textsuperscript{2+}i-dependent and-independent signalling pathways have been identified that stimulate the intracellular activation and/or release of proteases. The cell-permeant inhibitor of the Ca\textsuperscript{2+}i-dependent protease calpain, Calpain Inhibitor I, did not prevent high Ca\textsuperscript{2+}i-induced suppression of luciferase activity in the present study. Another candidate is the mitochondrial serine protease Omi/HtrA, which localises to the mitochondrial inter-membrane space in close proximity to 1\alpha-\textnormal{OHase}. Omi/HtrA was previously shown to be activated and released via a PP2A-dependent signalling pathway (83). The Ca\textsuperscript{2+}i-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\(^{2+}\) 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\(^{2+}\) concentration-dependent control of CYP27B1 promoter-dependent firefly luciferase activity in HEK-293 cells. At submaximal Ca\(^{2+}\), the CaSR mediates Ca\(^{2+}\)-stimulated activation of the CYP27B1 promoter dependent on two parallel signalling 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\(^{2+}\), the CaSR mediates a post-transcriptional mechanism, most probably protein breakdown, dependent upon a mechanism that links the CaSR to phosphorylation of CaSR residue T888 via PKC\(\alpha\) and to an ERK\(_{1/2}\)-dependent pathway, presumably downstream of CaSR\(^{T888}\) phosphorylation. The results demonstrate the existence of complex Ca\(^{2+}\)-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 extra-renal tissues, along with negative control of 1αOHase activity in the renal proximal tubule.
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Figure Legends

Fig. 1. Ca\(^{2+}\) concentration and time dependences of firefly and *Renilla* luciferase activity in co-transfected HEK-CaSR cells

HEK-CaSR and HEK-293 cells were transiently co-transfected with full length (1501 bp) CYP27B1-firefly luciferase reporter gene and the TK-*Renilla* luciferase control gene then incubated at various Ca\(^{2+}\) concentrations as shown for various times from 2-24 h 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 two further experiments. In (B) the data were obtained in three experiments and are expressed as Relative Luciferase Activities.

Fig. 2. Biphasic Ca\(^{2+}\) concentration-dependent control of relative luciferase activity in HEK-CaSR cells co-transfected with CYP27B1–firefly luciferase and control *Renilla* luciferase constructs

HEK-CaSR and HEK-293 cells were transiently co-transfected with full length (1501 bp) CYP27B1-firefly luciferase reporter gene and the TK-*Renilla* luciferase control gene then incubated for 24 h at various Ca\(^{2+}\) concentrations prior to analysis for both firefly and *Renilla* luciferases. The results show the impacts of Ca\(^{2+}\) 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 6 independent experiments in C. Luciferase activities were corrected for the background levels obtained in untransfected cells. Some errors lie entirely within the symbols.

Fig. 3. The CaSR mediates biphasic Ca\(^{2+}\) concentration-dependent control of CYP27B1 luciferase expression
Luciferase assays were performed on HEK-CaSR cells transiently co-transfected 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 h in 0.2% BSA supplemented DMEM in the presence of various Ca\(^{2+}\), in the absence or presence of (A) cinacalcet (1 µM) or (B) NPS-2143 (1 µM). Firefly luciferase activities were corrected for background and expressed relative to Renilla luciferase activities. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared to 0.5 mM Ca\(^{2+}\). †, p < 0.05; ††, p < 0.01 compared to 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. U, untransfected HEK-CaSR control cells.

**Fig. 4.** Ca\(^{2+}\) stimulated firefly luciferase and 1\(\alpha\)OHase mRNA expression in transfected HEK-CaSR cells

Quantitative RT-PCR for (A) firefly luciferase expression was performed on HEK-CaSR cells transiently co-transfected 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 h in 0.2% BSA supplemented DMEM at 0.5, 3.0, or 5.0 mM Ca\(^{2+}\). (B) 1\(\alpha\)OHase expression was also quantified in untransfected HEK-CaSR cells and (C) HEK-293 cells, as described, for 0.5, 3.0, 5.0, or 7.0 mM Ca\(^{2+}\). mRNA expression levels were expressed as fold change with respect to the 0.5 mM Ca\(^{2+}\) control, relative to RNA polymerase II using the \(2^{-\Delta\Delta C_T}\) method. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared to 0.5 mM Ca\(^{2+}\) treated cells using one-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.

**Fig. 5.** High Ca\(^{2+}\)-induced inhibition of CYP27B1-firefly luciferase: requirement for protein kinase C phosphorylation of CaSR C-terminus
(A) Effect of PKC inhibitor, GF109203X, on \( \text{Ca}^{2+}_o \)-dependent CYP27B1-firefly luciferase. Luciferase assays were performed on HEK-CaSR cells transiently co-transfected 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 h in 0.2% BSA supplemented DMEM at 0.5 mM \( \text{Ca}^{2+}_o \) in the presence of vehicle (0.1% DMSO), GF109203X (GFX; 0.5 or 1.0 \( \mu \text{M} \)). The \( \text{Ca}^{2+}_o \) concentration was then either maintained at 0.5 mM or increased to 3.0 or 5.0 mM for 24 h. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. *, \( p < 0.01 \); **, \( p < 0.001 \) compared to 0.5 mM \( \text{Ca}^{2+}_o \); †, \( p < 0.05 \) compared to 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 co-transfected 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 \( \text{Ca}^{2+}_o \) in the absence or presence of cinacalcet for 24 h. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. *, \( p < 0.05 \); **, \( p < 0.001 \) compared to 0.5 mM \( \text{Ca}^{2+}_o \); †, \( p < 0.01 \) compared to absence of cinacalcet; ‡, \( p < 0.05 \); ‡‡, \( p < 0.01 \); ‡‡‡, \( p < 0.001 \) compared to wild type CaSR. The data were obtained in 6 independent experiments performed in triplicate.

**Fig. 6. Impact of \( \text{Ca}^{2+}_o \) 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 5.0 mM \( \text{Ca}^{2+}_o \) for 24 h, followed by western blotting. Over the extended period of the experiment we did not observe high \( \text{Ca}^{2+}_o \)-induced
dephosphorylation of CaSR-T888. Representative western blot from 6 independent experiments. (B) Quantitation of western blot intensity data. In response to either 3.0 mM or 5.0 mM Ca\textsuperscript{2+} a stable increase in pCaSR\textsuperscript{T888} was observed for the 140 kDa (high mannose) form but not the 160 kDa (complex glycosylated) form.

**Fig. 7. Effect of ERK inhibition and combined PKC/ERK inhibition on biphasic Ca\textsuperscript{2+} concentration-dependent control of CYP27B1-firefly luciferase**

(A) Effect of MEK inhibitor, PD184352, on Ca\textsuperscript{2+}-dependent CYP27B1 –1501 bp promoter-controlled firefly luciferase expression in transfected HEK-CaSR cells. Luciferase assays were performed on HEK-CaSR cells transiently co-transfected with the firefly luciferase reporter gene (CYP27B1 –1501 bp promoter), and the Renilla luciferase gene. Cells were preincubated 1 h in 0.2% BSA supplemented DMEM at 0.5 mM Ca\textsuperscript{2+} in the absence or presence of PD184352 (10 µM). The Ca\textsuperscript{2+} concentration was then either maintained at 0.5 mM or increased up to various concentrations (from 3.0 to 9.0 mM) for 24 h. Firefly luciferase activities were corrected for background and expressed relative to Renilla luciferase activities. *, p < 0.01; **, p < 0.001 compared to 0.5 mM Ca\textsuperscript{2+}. The data were obtained in 5 independent experiments, performed in triplicate.

(B) Effects of PKC and MEK inhibitors alone and in combination on Ca\textsuperscript{2+}-dependent CYP27B1 promoter-dependent luciferase activity. Luciferase assays were performed on HEK-CaSR cells transiently co-transfected with the firefly luciferase reporter gene (CYP27B1 –1501 bp promoter), and the Renilla luciferase gene. Cells were preincubated 1 h in 0.2% BSA supplemented DMEM at 0.5 mM Ca\textsuperscript{2+} in the presence of vehicle (0.2% DMSO), or GF109203X (1 µM) or PD184352 (10 µM), or both these compounds. The Ca\textsuperscript{2+} concentration was then either maintained at 0.5 mM or increased to 3.0 or 5.0 mM for 24 h. Firefly luciferase activities were corrected for background and
expressed relative to *Renilla* luciferase activities. *, p < 0.01; **, p < 0.001 compared to 0.5 mM Ca$^{2+}$; †, p < 0.01; ††, p < 0.001 compared to vehicle (0.2% DMSO). The data were obtained in 4 independent experiments performed in triplicate.

Fig. 8. Impacts of selected mutations of response elements in the −102 bp CYP27B1 promoter on Ca$^{2+}$-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). They include: 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 co-transfected 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 h in 0.2% BSA supplemented DMEM at 0.5 and 3.0 mM Ca$^{2+}$. Firefly luciferase activities were corrected for background and expressed relative to *Renilla* luciferase activities. *, p < 0.001 compared to 0.5 mM Ca$^{2+}$; †, p < 0.001 compared to −305 bp promoter. The data were obtained in 6 independent experiments performed in triplicate.

Statement on data availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in
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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

Luciferase assays were performed on HEK-CaSR cells transiently co-transfected 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 h in 0.2% BSA supplemented DMEM at 0.5 and 3.0 mM Ca\(^ {2+}\)\(_o\). Firefly luciferase activities were corrected for background and expressed relative to Renilla luciferase activities. *, p < 0.01; **, p < 0.001 compared to –1501 bp. RLA, Relative Luciferase Activity.

| Promoter Construct | Relative Luciferase Activity (RLA) | RLA (Fold Change) | n  |
|--------------------|-----------------------------------|-------------------|----|
| –1501 bp           | 8.2 ± 0.6                         | 2.6 ± 0.3         | 10 |
| –305 bp            | 6.4 ± 0.7                         | 2.4 ± 0.4         | 10 |
| –200 bp            | 19.3 ± 3.6*                       | 1.8 ± 0.4         | 5  |
| –102 bp            | 5.3 ± 1.0                         | 1.9 ± 0.5         | 10 |
Fig. 1.
Fig. 2.

A

B

C

Firefly Luciferase Counts ($\times 10^4$)

Ca$^{2+}$ concentration (mM)

Relative Luciferase Activity

Ca$^{2+}$ concentration (mM)

Ca$^{2+}$ concentration (mM)
Fig. 3.

A

B

C

0.5 3.0 0.5 3.0
0
10
20
30
40
Ca\textsuperscript{2+} concentration (mM)
Relative Luciferase Activity

1 \mu M Cinacalcet

†

**

* ††

0.5 3.0 5.0 7.0 0.5 3.0 5.0 7.0
0
10
20
30
40
Ca\textsuperscript{2+} concentration (mM)
Relative Luciferase Activity

1 \mu M NPS-2143

***

††

†

1 \mu M Cinacalcet

1 \mu M NPS-2143

U 0.5 3.0 5.0 0.5 3.0 5.0

Ca\textsuperscript{2+} Concentration (mM)

Luciferase

GAPDH
Fig. 4.

A. Firefly Luciferase mRNA Levels (Normalised to RNA Pol II)

B. 1αOHase mRNA Levels (Normalised to RNA Pol II)

C. 1αOHase mRNA Levels (Normalised to RNA Pol II)

Ca^{2+} concentration (mM)
Fig. 5.

A

Vehicle 0.5 µM GFX 1 µM GFX

**

B

HEK-CaSR WT HEK-CaSR T888A

1 µM Cinacalcet 1 µM Cinacalcet

** †

‡

‡* ‡‡‡ ‡‡

‡‡
Fig. 6.

A

- **Ca\textsuperscript{2+} Concentration (mM)**
  - 0.5
  - 3.0
  - 5.0

- **kDa**
  - 160
  - 140

- **pCaSR T888**
- **Total CaSR**

B

- **pCaSR \textsuperscript{T888} / Total (160-kDa)**
- **pCaSR \textsuperscript{T888} / Total (140-kDa)**

- **Ca\textsuperscript{2+} Concentration (mM)**
  - 0.5
  - 3.0
  - 5.0

- **pCaSR**
  - NS
  - *

- **Total CaSR**
  - NS
Fig. 7.

A

![Graph showing relative luciferase activity with different Ca\(^{2+}\) concentrations for Vehicle and 10 µM PD184352 conditions.](image)

B

![Graph showing relative luciferase activity with different treatments: Vehicle, 1 µM GF109203X, 10 µM PD184352, and 1 µM GF109203X + 10 µM PD184352.](image)
Fig. 8.

A

B

Relative Luciferase Activity

Ca^{2+}_o concentration (mM)

-305 bp

-102 bp

WT

mCCAAT

mSp1(p)