Calcitonin stimulates expression of the rat 25-hydroxyvitamin D₃-24-hydroxylase (CYP24) promoter in HEK-293 cells expressing calcitonin receptor: identification of signaling pathways

X-H Gao, P P Dwivedi, J L Omdahl, H A Morris and B K May

School of Molecular and Biomedical Science, University of Adelaide, Adelaide 5005, South Australia, Australia

1Department of Biochemistry and Molecular Biology, University of New Mexico, School of Medicine, Albuquerque, New Mexico 87131–5221, USA

2Hanson Institute, Adelaide 5005, South Australia, Australia

(Requests for offprints should be addressed to B K May; Email: brian.may@adelaide.edu.au)

Abstract

Regulation of the gene for renal 25-hydroxyvitamin D-24-hydroxylase (CYP24) is important for controlling the level of circulating 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). We report here for the first time that the peptide hormone calcitonin significantly stimulates expression of a rat CYP24 promoter-luciferase construct in both transiently and stably transfected kidney HEK-293 cells. A GC box at −114/−101 and a CCAAT box at −62/−51 have been identified that underlie both basal expression of the CYP24 promoter and the calcitonin inductive response. Data from overexpression studies suggested that Sp1 and NF-Y are the proteins that function through the GC and CCAAT boxes respectively. ERK1/2 signaling pathways were not involved in the calcitonin-mediated response, since stimulation of the promoter was unaffected by the pharmacological ERK1/2 inhibitor PD98059 and by a dominant negative mutant of ERK1/2 (ERK1K71R). In contrast, calcitonin induction but not basal expression was dependent on protein kinase A and protein kinase C (PKC) activities with the inhibitors H89 and calphostin C lowering induction by 50–60%. The atypical PKC, PKCζ contributes to calcitonin induction, but not to basal expression of the CYP24 promoter, since overexpression of a dominant negative clone PKCζK281 M lowered induction by 50%. Cotransfection of a dominant negative form of Ras resulted in calcitonin-mediated induction being reduced also by about 50%. A Ras–PKCζ signaling pathway for calcitonin action is proposed, which acts through the GC box. The findings have been extrapolated to the in vivo situation where we suggest that induction of renal CYP24 by calcitonin could be important under hypercalcemic conditions thus contributing to the lowering of circulating 1,25(OH)₂D₃ levels.


Introduction

The ambient level of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) represents a balance between its metabolic activation and inactivation (Omdahl & May 1997, Jones et al. 1998, Omdahl et al. 2002). Bioactivation occurs predominantly in the kidney where the mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D₃ 1α-hydroxylase (CYP27B1) catalyzes the addition of a hydroxyl group at C-1 on 25-hydroxyvitamin D₃ to generate the hormonally active 1,25(OH)₂D₃ (Jones et al. 1998, Omdahl et al. 2002). Inactivation of 1,25(OH)₂D₃ occurs via the C-23/C-24 oxidation pathways catalyzed by the multifunctional mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D₃ 24-hydroxylase or CYP24 present in the kidney and most other tissues (Jones et al. 1998, Brown et al. 1999). CYP24 initiates the inactivation and degradation of 1,25(OH)₂D₃ through side-chain hydroxylation at either C-23 or C-24, depending on the species (Omdahl et al. 2002). While 1,25(OH)₂D₃ can be synthesized at extra-renal sites (Panda et al. 2001, Segersten et al. 2002) where it likely acts in a paracrine/autocrine fashion, the production of 1,25(OH)₂D₃ by the
kidney is accepted as the primary source of endocrine-acting 1,25(OH)₂D₃.

Circulating 1,25(OH)₂D₃ together with the peptide hormone, parathyroid hormone (PTH), are the key regulators of ambient calcium levels that must be controlled to support bone mineralization and other cellular processes. Small fluctuations in serum calcium are detected by the calcium-sensing receptor present on the cell surface of the PTH-secreting parathyroid cells, with a lowered level of calcium resulting in increased PTH production (Brown 2000, Brown & MacLeod 2001). In turn, PTH through the PTH receptor located in the kidney cell plasma membrane up-regulates expression of renal CYP27B1 and hence 1,25(OH)₂D₃ synthesis (Jones et al. 1998, Omdahl et al. 2002). The resultant increased levels of PTH and 1,25(OH)₂D₃ lead to increased serum calcium through enhanced kidney calcium re-absorption, stimulated bone resorption and intestinal calcium uptake (Brown & MacLeod 2001).

A second peptide hormone, calcitonin, secreted by the thyroid parafollicular C cells has been considered as a possible contributor to the maintenance of serum calcium (Pondel 2000, Inzerillo et al. 2002). Production of calcitonin by these cells in response to calcium differs fundamentally from that of PTH with increased serum calcium levels detected by the calcium-sensing receptor resulting in the stimulation of calcitonin production (Brown & MacLeod 2001). Calcitonin has hypocalcemic actions and is well known to inhibit osteoclastic bone resorption and stimulate renal calcium excretion in human and rodents (Jones et al. 1998, Pondel 2000). However, it is uncertain as to whether these actions of calcitonin contribute significantly to the maintenance of ambient calcium levels in human adults (Brown & MacLeod 2001).

Alternatively, calcitonin could contribute to calcium homeostasis through regulating 1,25(OH)₂D₃ levels. In this regard, Shinki et al. (1999) have presented evidence from experiments involving hormone administration to rats on dietary calcium, that calcitonin but not PTH induces renal CYP27B1 under conditions of normal or high serum calcium. Administered PTH on the other hand induced CYP27B1 only under hypocalcemic conditions. In other experiments Murayama et al. (1999) showed that calcitonin or PTH treatment of normal rats increased CYP27B1 mRNA production in the kidney. In vivo experiments such as these are inherently difficult to interpret because in rodents administration of pharmacological doses of calcitonin will result in hypocalcemia with subsequent stimulation of PTH production. Thus such experiments require parathyroid gland removal and hormone replacement.

In other studies employing transfected kidney cells, it has been shown that the promoter for human CYP27B1 is up-regulated by calcitonin and also PTH (Murayama et al. 1998, 1999). In spite of the evidence for calcitonin-stimulated expression of renal CYP27B1, no plausible physiological role for this finding has been proposed. There is no information on the regulation of the CYP24 gene expression in kidney following calcitonin treatment although Beckman et al. (1994) reported its suppression of CYP24 mRNA in the intestine of rats.

We have investigated whether rat CYP24 promoter activity can be altered by calcitonin in transfected kidney cells. We show for the first time that calcitonin stimulates expression of the rat CYP24 promoter and we have identified signal pathways and responsive promoter control elements. We suggest that the induction of CYP24 by calcitonin may have physiological relevance in the hypercalcemic state.

Materials and methods

Materials

Salmon calcitonin was obtained from Peninsula Laboratory, Inc. (Belmont, CA, USA); 1,25(OH)₂D₃ was purchased from Tetrionics, Inc. (Madison, WI, USA); H89, calphostin C and PD98059 were from BioMol Research Laboratories (Plymouth Meeting, PA, USA); DOTAP was from Roche Diagnostics Corp. (Indianapolis, IN, USA); the luciferase assay kit was from Promega (Madison, WI, USA); [α-³²P]dCTP (10 µCi/µl) was purchased from Geneworks (Adelaide, SA, Australia); [α-³²P]dCTP (10 µCi/µl) was purchased from Geneworks.

Promoter luciferase constructs and expression plasmids

The CYP24 promoter luciferase construct used in this study (pCYP24WT-Luc) contains −298 bp of
rat CYP24 promoter sequence together with 74 bp of 5′-untranslated region in the pGL3 basic vector together with the firefly luciferase reporter gene as described previously (Dwivedi et al. 2000). The mutant constructs, pCYP24-mVDRE1+2-Luc and pCYP24-mEBS-Luc (VDRE=vitamin D response element; EBS=Ets-1 binding site), have also been described (Dwivedi et al. 2000).

A dominant negative form of NF-Y, NF-YAm29 was kindly provided by Dr R Mantovani (University of Milan, Italy); a dominant negative form of ERK1, ERK1K71R, was from Dr C Hii (Women and Children’s Hospital, Adelaide, SA, Australia); the dominant negative mutant of PKCζ, PKCζK281 M, was from Dr J W Soh (Columbia University, NY, USA).

Site-directed mutagenesis
Site-directed mutagenesis of the CCAAT box and GC box sequences localized in the rat CYP24 promoter was carried out using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA) according to the manufacturer’s instruction. The mutagenesis primers were designed as follows (the core sequence is underlined and the mutated oligonucleotides are in bold letters). wtCCAAT, 5′-AGCGTGCTCATTGGCCACTCCAGC-3′; mCCAAT, 5′-CAGCGTGCCACTCCAGC-3′; wtGC, 5′-CTTCCACACCCCGCCCCGCGTCCCTC-3′; mGC, 5′-CTTCCCACACCCCGAACCCCGCAGTCCCTC-3′.

Cell culture and transient transfection
HEK-293 cells stably expressing the insert-negative form of calcitonin receptor (CTR), and designated as HR-12 cells, were kindly provided by Dr David Findlay (Department of Orthopedics and Trauma, University of Adelaide, SA, Australia) as reported (Evdokiou et al. 2000, Raggatt et al. 2000). HR-12 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal calf serum and 200 µg/ml G418. HR-12 cells (1 × 10⁶ cells) were seeded into one 60 mm Petri dish with 5 ml DMEM medium with 10% fetal calf serum and 200 µg/ml G418. Once attached, the cells were transfected with the cocktail of 50 µl DNA mixture (10 µg promoter construct, 100 ng pEF-IRES-Puro vector kindly provided by Professor John Wallace, University of Adelaide, Adelaide, SA, Australia) and 50 µl DOTAP mixture (30 µg DOTAP). The 100 µl DNA/DOTAP cocktail was left at room temperature for 20 min before being transfected into the cells. The transfected cells were grown at 37 °C for about 24 h until almost confluent and then subcultured into two 100 mm Petri dishes with 8 ml of culture medium for a further 24 h. The medium was replaced with fresh medium containing 1 µg/ml puromycin for 4–7 days or until the medium color changed to yellow. The medium was replaced again and the cells were left for another 4–7 days. Finally, the cells were pooled into one 25 cm² flask with the selection medium containing 1 µg/ml puromycin and grown until confluency was reached. Pooled stably transfected cells (2 × 10⁶) were seeded into 24-well plates in 400 µl DMEM containing 10% fetal calf serum and 1 µg/ml puromycin and were allowed to grow overnight. Cells were treated with 10 nM calcitonin containing 10% fetal calf serum to achieve 60–70% confluence and the cells were allowed to attach for 6–7 h. Within an experiment, transfections were performed in triplicate and the data averaged; each transfection was performed using 200 ng CYP24 promoter luciferase construct. All experiments were repeated on at least two separate occasions. Data shown are from one representative experiment. Overexpression plasmids and dominant negative mutants were also cotransfected wherever indicated. All plasmid DNA was diluted to a final volume of 5 µl and mixed with 1.5 µg DOTAP diluted in 5 µl Hepes buffer (20 mM, pH 7.4). DNA-DOTAP complex formation was achieved by incubating the mixture for 15–20 min at room temperature and 10 µl of the complex were added to the cells. After transfection overnight, the medium was replaced with serum-free RPMI 1640 medium. Cells were treated with either H89, calphostin C or PD98059 for 1.5 h and then treated with calcitonin for 24 h before harvesting.

Generation of stably transfected cells
HR-12 cells (1 × 10⁶ cells) were seeded into one 60 mm Petri dish with 5 ml DMEM medium with 10% fetal calf serum and 200 µg/ml G418. Once attached, the cells were transfected with the cocktail of 50 µl DNA mixture (10 µg promoter construct, 100 ng pEF-IRES-Puro vector kindly provided by Professor John Wallace, University of Adelaide, Adelaide, SA, Australia) and 50 µl DOTAP mixture (30 µg DOTAP). The 100 µl DNA/DOTAP cocktail was left at room temperature for 20 min before being transfected into the cells. The transfected cells were grown at 37 °C for about 24 h until almost confluent and then subcultured into two 100 mm Petri dishes with 8 ml of culture medium for a further 24 h. The medium was replaced with fresh medium containing 1 µg/ml puromycin for 4–7 days or until the medium color changed to yellow. The medium was replaced again and the cells were left for another 4–7 days. Finally, the cells were pooled into one 25 cm² flask with the selection medium containing 1 µg/ml puromycin and grown until confluency was reached. Pooled stably transfected cells (2 × 10⁶) were seeded into 24-well plates in 400 µl DMEM containing 10% fetal calf serum and 1 µg/ml puromycin and were allowed to grow overnight. Cells were treated with 10 nM calcitonin
in serum-free RPMI 1640 medium for 24 h. The luciferase activity from the stably transfected cells was normalized by the protein content in each sample.

**Luciferase assay**

Preliminary experiments showed that the dual luciferase assay previously used (Gao et al. 2002) could not be employed because the internal Renilla luciferase driven by the SV40 promoter was consistently induced at least 2-fold by calcitonin. Hence the level of firefly luciferase activity in cell lysates has been expressed relative to protein content. For determination of luciferase activity, the cells were lysed using 100 µl 1 × reporter assay buffer (Promega) for 20 min at 25 °C. Luciferase assay substrate (Promega) was added (25 µl) to 10 µl of cell lysate and firefly luciferase activity determined according to the Promega protocol. The protein content of the cell lysate was determined using 2 µl of supernatant and the protein microassay kit (Bio-Rad, NSW, Australia) according to the manufacturer’s instructions. BSA was used as the protein standard.

**Results**

**Calcitonin responsiveness of the rat CYP24 gene promoter**

The construct employed here (pCYP24WT-Luc) contains 298 bp of the rat CYP24 gene promoter together with 74 bp of 5′-untranslated region, cloned upstream of the firefly luciferase cDNA sequence as reporter. This region of the CYP24 promoter has been intensively studied and encompasses two VDREs at −258/−244 and −150/−136 and a functional EBS at −128/−119 (Kerry et al. 1996, Dwivedi et al. 2000). pCYP24WT-Luc was transiently transfected into HR-12 cells stably expressing the insert-negative isoform of the human CTR. The insert-negative isoform of the human CTR lacks a 16 amino acid insert present in the first intracellular loop of the other major expressed insert positive isoform and, as such, can activate phospholipase C (PLC), adenylyl cyclase (Moore et al. 1995, Sexton et al. 1999, Pondel 2000) and mitogen-activated protein (MAP) kinase ERK1/2 pathways (Chen et al. 1998, Raggatt et al. 2000). Transfected cells in serum-free medium were treated for 24 h with salmon calcitonin at 10 nM, the concentration used in previous studies (Evdokiou et al. 2000, Raggatt et al. 2000).

It can be seen in Fig. 1 that calcitonin induces pCYP24WT-Luc nearly 5-fold. Luciferase activities in all experiments have been corrected for the value of the pGL3 empty vector. A construct containing −1400 bp of 5′ flanking region of the rat CYP24 gene, the maximal length available, was also investigated and the level of calcitonin induction observed was similar to that for the −298 bp pCYP24WT-Luc construct, indicating that calcitonin responsive elements are likely to be confined to the first −298 bp of sequence.

**Identification of calcitonin responsive sites in the −298 bp promoter by transient transfections**

We have previously characterized a functional EBS located downstream of VDRE1 at −150/−136 in the rat CYP24 promoter (Hahn et al. 1994, Dwivedi et al. 2000). Using TFSEARCH Website (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl), we recently identified two more potential binding sites located further downstream, an inverted CCAAT box at −62/−51 (5′-GCTCATTGGCC A-3′) and a GC box at −114/−101 (5′-ACACC CGCCCCCGG-3′) (Fig. 1). Site-directed mutagenesis showed that the EBS did not contribute to basal expression of pCYP24WT-Luc, but the GC and CCAAT box sequences were particularly important with inactivation of either site in the constructs pCYP24mGC-Luc and pCYP24mCCAAT-Luc, markedly lowering basal expression from 1·05 ± 0·14 to 0·33 ± 0·04 and 0·14 ± 0·01 respectively (Fig. 1). The expression level was 0·13 ± 0·03 when both the GC and CCAAT box sites were mutated together (Fig. 1). When the two VDREs were mutated simultaneously in pCYP24mVDRE1+2-Luc, an increase in basal expression was seen (from 1·05 ± 0·14 to 1·97 ± 0·29) (Fig. 1). Such an increase has been previously observed in COS-1 cells and reflects promoter repression by unliganded vitamin D receptor (VDR)/retinoid X receptor (RXR) bound to the VDREs (Dwivedi et al. 1998, Polly et al. 2000).

An examination of these mutant constructs in the presence of calcitonin revealed that while the...
EBS and VDRE sites were essentially unresponsive, both the GC and CCAAT boxes contributed to calcitonin stimulation with inactivation of each site lowering induction by about 50% (from $4.67 \pm 0.29$ to $2.53 \pm 0.46$- and $2.57 \pm 0.38$-fold respectively) (Fig. 1). Mutagenesis of both the GC and CCAAT sites in $pCYP24mGC+CCAAT-Luc$ further lowered the fold induction by calcitonin to $1.76 \pm 0.17$ (Fig. 1). Thus the CCAAT and GC boxes are key elements underlying calcitonin responsiveness.

**Functional role for the GC and CCAAT box in stably transfected cells**

The functional roles of the GC and CCAAT box elements were also investigated in pooled HR-12 cells stably expressing the wild type ($pCYP24WT-Luc$) or mutated constructs ($pCYP24mGC-Luc$ and $pCYP24mCCAAT-Luc$) in the presence of serum-free medium.

Calcitonin induced $pCYP24WT-Luc$ by $14.39 \pm 0.99$-fold, a level higher than that observed in transiently transfected cells. Mutagenesis of either the GC box or the CCAAT box lowered basal expression from $3.38 \pm 0.67$ to $1.64 \pm 0.41$ and $0.33 \pm 0.07$ respectively. Calcitonin induction was markedly reduced with inactivation of either the GC or CCAAT box sites lowering expression by 80 and 95% respectively, from $14.39 \pm 0.99$ to $2.79 \pm 0.34$ and $1.22 \pm 0.09$. Mutagenesis of both sites together was not examined in stably transfected cells. Hence, the GC and CCAAT box sites are major contributors to calcitonin inductions when stably integrated into the chromosome, as found in transient transfection analysis.

**Characterization of the GC and CCAAT box binding protein**

Overexpression of Sp1 in HR-12 cells did not alter the fold level of calcitonin induction of $pCYP24WT-Luc$, most likely reflecting a high level of endogenous Sp1. However, overexpression of Sp1 markedly increased expression of this construct about 350-fold in transiently transfected *Drosophila* SL3 cells that lack endogenous Sp1 and this stimulation occurred at least in part through the GC box (data not shown).

The sequence, orientation and position of the inverted CCAAT box sequence at $-62/-51$, conforms to those sites shown to bind
the ubiquitous transcription factor NF-Y that constitutes a trimer of NF-YA, B and C subunits (Mantovani 1999). To determine if NF-Y plays a role in CYP24 promoter expression, NF-YA was overexpressed in HR-12 cells together with pCYP24WT-Luc, but no effect on promoter activity was seen. Overexpression of NF-YA, B and C together in Drosophila SL3 cells, which lack NF-Y, increased expression of the promoter by about 7-fold. This action was mediated entirely through the CCAAT box (data not shown).

The dominant negative NF-YA mutant NF-YAm29 (Mantovani et al. 1994) was expressed in HR-12 cells at 200 ng. Under such conditions, basal expression of the pCYP24WT-Luc construct was shown to be strongly inhibited from 0.23 ± 0.01 to 0.06 ± 0.01, while calcitonin-mediated fold induction was moderately lowered from 5.33 ± 0.30-fold to 3.59 ± 0.38-fold (Fig. 2). However, at 200 ng NF-YAm29, basal expression of pCYP24mCCAAT-Luc was unaltered (from 0.03 ± 0.01 to 0.03 ± 0.01) while calcitonin-mediated fold induction was not altered significantly (compare 2.57 ± 0.38 with 2.84 ± 0.52). The data suggest that NF-Y acting through the CCAAT box plays a role in both basal and calcitonin induced expression of pCYP24WT-Luc.

**ERK1/2 pathways and calcitonin stimulation**

In a previous study, it was established that treatment of HR-12 cells with calcitonin at 10 nM resulted in a marked activation of ERK1/2 activity that could be inhibited by the ERK1/2 kinase inhibitor PD98059 at 50 µM (Raggatt et al. 2000). The mechanism of this ERK1/2 activation is not known, but it is likely to be protein kinase C (PKC)-dependent (Chabre et al. 1992). We speculated that ERK1/2 activity may participate in calcitonin stimulation of the −298 bp CYP24
promoter particularly since our recent work has shown that this activity is important for induction of this promoter by 1,25(OH)₂D₃ (Dwivedi et al. 2002). However, our data do not provide evidence for the involvement of ERK1/2. First, PD98059 at 50 µM had no effect on basal or calcitonin-induced expression of pCYP24₃WT-Luc with fold induction in the presence of calcitonin (4·67 ± 0·29) not affected by the inhibitor (4·42 ± 0·42).

Furthermore, overexpression of the ERK1/2 dominant negative clone, ERK1K71R (200 ng), in HR-12 cells had no effect on basal expression or calcitonin induction of pCYP24₃WT-Luc measured after 24 h (the fold induction in the absence and presence of the mutant being 4·67 ± 29 and 4·16 ± 0·27 respectively). In a control experiment, the inhibitory effect of this mutant clone was confirmed with induction of pCYP24₃WT-Luc by 1,25(OH)₂D₃ at 100 nM measured after 24 h being lowered by ERK1K71R (from 49·24 ± 3·90-fold to 32·87 ± 3·74), in keeping with results reported in COS-1 cells (Dwivedi et al. 2002).

**Involvement of protein kinase A (PKA), PKC, Ras and PKCCζ in calcitonin stimulation**

It is well known that the activated insert-negative isoform of the human CTR can couple to PKA and PKC pathways in a G protein-dependent way (Chen et al. 1998, Shyu et al. 1999, Pondel 2000). The data in Fig. 3 show that the PKA inhibitor, H89, and the PKC inhibitor, calphostin C, did not affect basal activity of pCYP24₃WT-Luc in HR-12 cells. However, H89 and calphostin C markedly inhibited calcitonin-induced expression by 60% (from 4·88 ± 0·44-fold to 1·96 ± 0·07-fold) and 50% (from 4·88 ± 0·44-fold to 2·50 ± 0·13-fold) respectively (Fig. 3). Hence PKA and PKC activities play a role in calcitonin-induced expression but not basal expression.

In apparent support for PKA involvement, the PKA activator forskolin at 10 µM moderately stimulated basal expression of pCYP24₃WT-Luc in HR-12 cells by 2·46 ± 0·49. When the CCAAT box and GC box were mutated individually, the fold induction by forskolin was lowered to 1·51 ± 0·36 and 1·40 ± 0·18 respectively, with mutagenesis of both sites giving 1·16 ± 0·15-fold induction. Hence both the CCAAT and GC boxes appear to be target sites for PKA signaling.

The atypical PKC isof orm PKCζ can bind and phosphorylate the zinc finger region of Sp1 (Pal et al. 1998). Since our evidence implicates Sp1 in both basal and calcitonin induction of pCYP24₃WT(298)-Luc in HR-12 cells, it was of interest to investigate the possible involvement of PKCζ. A dominant negative clone of PKCζ, PKCζK281 M (Soh et al. 1999), when overexpressed at 200 ng did not alter basal expression of pCYP24₃WT-Luc in HR-12 cells (data not shown). However, this mutant clone consistently inhibited calcitonin induction by about 55% with an approximate 5·5-fold level of induction being reduced to about 2·4-fold and the inhibitory action of the mutant clone was dependent on a functional GC box (Fig. 4). Hence, phosphorylation of Sp1 may be needed for calcitonin induction but not for basal expression of the CYP24 promoter. An involvement of Ras was also demonstrated with overexpression of the Ras dominant negative mutant (Ras 17N) at 200 ng inhibiting induction of pCYP24₃WT-Luc about 50% (from about 5·5-fold to 2·1-fold) as shown in Fig. 4. Basal expression was not altered. When the GC box was mutated, overexpression of Ras 17N did not further lower calcitonin induction showing that the dominant clone acts solely through the GC box (Fig. 4).
Synergistic effect of calcitonin and 1,25(OH)₂D₃ on CYP24 promoter activity

Since 1,25(OH)₂D₃ (Hahn et al. 1994, Kerry et al. 1996) and calcitonin (this study) markedly up-regulate CYP24 promoter expression, it was of interest to investigate the additive action of both hormones. A marked synergistic response was observed (Fig. 5). Calcitonin alone stimulates about 4.7-fold, while 1,25(OH)₂D₃ stimulates 34-fold, but together there is about a 117-fold increase in promoter activity. Synergy was abolished when the two VDREs were mutated (Fig. 5). However, a high level of synergy was maintained when the calcitonin responsive CCAAT and GC boxes were mutated together (Fig. 5). Hence the synergistic action is dependent on the VDREs. A high level of transcriptional synergy was also observed in cells stably transfected with pCYP24WT(298)-Luc and was dependent on the two VDREs (data not shown). Since 1,25(OH)₂D₃ stimulation of the CYP24 promoter involves ERK1/2 activity, it seems highly likely that the synergy results from enhanced activation of these ERKs by calcitonin.

Discussion

In this study, it has been shown for the first time that the −298 bp promoter region of the rat CYP24 gene can be significantly induced by calcitonin at 10 nM. When HR-12 cells expressing the CTR were transiently transfected with pCYP24WT(298)-Luc or pCYP24mGC(298)-Luc constructs with or without 500 ng PKCζK281 M and incubated with 10 nM calcitonin. The results are present as fold induction, the ratio of luciferase activity from calcitonin-treated cells to that from untreated cells (basal) representing the mean±S.D. of triplicate samples. Data are representative of two independent experiments.
confirm that these proteins function in vivo at these sites. The only transcription factor so far implicated in calcitonin action is Sp1, with calcitonin activation of the human p21 gene promoter showing a dependency on Sp1 binding sites (Evdokiou et al. 2000). Hence, the involvement in calcitonin-mediated induction reported here of a CCAAT box binding protein, most likely NF-Y, represents only the second target transcription factor to be identified.

Previous studies have reported that calcitonin treatment of HR-12 cells leads to a marked and prolonged activation of ERK1/2 (Raggatt et al. 2000). However, we show here that calcitonin induction of the CYP24 promoter is independent of ERK1/2 activity, since neither the ERK1/2 inhibitor PD98059 nor the dominant negative mutant ERK1K71R affected calcitonin induction. Experiments with specific inhibitors for PKA (H89) and PKC (calphostin C) provided evidence for the involvement of these signaling pathways in calcitonin induction but not basal expression. In the presence of forskolin, basal promoter expression was somewhat stimulated with both the GC and CCAAT box sites being targets. This finding supported the idea that one action of calcitonin is to increase PKA activity and hence promoter expression through both the CCAAT and GC boxes. Perhaps phosphorylation of Sp1 and NF-Y enhances their activity.
The role of PKC in calcitonin induction remains unclear, but one possibility is that it is involved in the activation of Ras, phosphatidylinositol 3-kinase (PI-3K) and hence PKC as described in other cells (Rodriguez-Viciana et al. 1994, 1996, Vanhaesebroeck & Alessi 2000). PKC has received considerable attention, since it interacts with and phosphorylates Sp1 and may lead to its activation (Pal et al. 2001, Rafty & Khachigian 2001). A kinase-inactive mutant form of PKC, PKC\(_{\beta}\) K281 M (Soh et al. 1999) was employed to investigate whether this isoform plays a role in CYP24 promoter expression. Blocking endogenous PKC\(_{\beta}\) with this dominant negative mutant had no effect on basal activity but consistently inhibited calcitonin induction and evidence indicated that this action occurred through the GC site. It is apparent from these studies that PKC\(_{\beta}\)-dependent phosphorylation of Sp1 is important for calcitonin induction but not for basal activity. However, future studies are required to define the pathway of activation that lies between CTR, PKC and PKC\(_{\beta}\).

A speculative model for calcitonin induction of the CYP24 promoter is shown in Fig. 6. We suggest that in the absence of calcitonin, basal expression of the CYP24 promoter is low and dependent on Sp1 bound at the GC box and also NF-Y bound at the CCAAT box but not the EBS, whose action is sterically inhibited by a corepressor located on the VDRE (Dwivedi et al. 1998). It is proposed that calcitonin through a G protein coupled to the CTR results in the activation of PKA, which may then phosphorylate Sp1 and NF-Y enhancing their transactivation ability. It is further proposed that activation of PKC occurs through another G protein coupled to the CTR by a mechanism involving PLC\(\beta\) and diacylglycerol (DAG) (Chabre et al. 1992). PKC could then activate Ras and in turn the Ras–PI-3K–PDK-1–PKC\(_{\beta}\) signaling pathway.

![Figure 6](https://www.endocrinology.org)
leading to the phosphorylation and activation of Sp1. However, the data presented are preliminary and implicate only PKC, Ras and PKCζ and further work is required to verify this model.

It is interesting to speculate on the possible physiological significance of the finding that calcitonin stimulates CYP24 promoter expression. It is tempting to suggest that calcitonin in vivo contributes to calcium homeostasis through regulating serum 1,25(OH)2D3 levels. It is well accepted that 1,25(OH)2D3 synthesized by the kidney is important for maintenance of serum calcium (Jones et al. 1998, Omdahl et al. 2002). Hence in response to calcitonin, an elevated renal CYP24 production would lower the production of 1,25(OH)2D3 by the kidney. Such an action of calcitonin could be important in the hypercalcemic state when a transient increase in serum calcium triggers the production of calcitonin by the thyroid C cells (Brown & MacLeod 2001).

While calcitonin induces CYP24 expression in kidney cells in the current work, there is another report from transient transfection assays that this hormone also induces the CYP27B1 promoter (Murayama et al. 1998). A situation in which both CYP27B1 and CYP24 activities are induced by calcitonin seems paradoxical. However, we have shown in this study that calcitonin and 1,25(OH)2D3 together result in a large synergistic activation of CYP24 in kidney cells. This synergy could be responsible in vivo for ensuring that serum 1,25(OH)2D3 levels are kept low in response to calcitonin production in the hypercalcemic state. At the molecular level, synergy could involve elevated ERK1/2 activity in the presence of calcitonin, which would then be expected to further enhance stimulation of the CYP24 promoter by 1,25(OH)2D3 (Dwivedi et al. 2002).

In summary, it has been established that the CYP24 gene promoter can be up-regulated in kidney cells by calcitonin, and this action can be further enhanced in the presence of 1,25(OH)2D3. It is proposed that this regulation could be part of the process in which calcitonin regulates circulating calcium through controlling the production of renal 1,25(OH)2D3. The studies here have focused on promoter analysis in transient and stably transfected cells and in the future it will be important to determine if cellular mRNA levels in the presence of 1,25(OH)2D3 and calcitonin correlate with the promoter activities.

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