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

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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, PKC ζ contributes to calcitonin induction, but not to basal expression of the *CYP24* promoter, since overexpression of a dominant negative clone PKC ζ K281 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–PKC ζ 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.

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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 1 α -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 $1,25(\text{OH})_2\text{D}_3$.

Circulating $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ synthesis (Jones *et al.* 1998, Omdahl *et al.* 2002). The resultant increased levels of PTH and $1,25(\text{OH})_2\text{D}_3$ lead to increased serum calcium through enhanced kidney calcium reabsorption, 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 $1,25(\text{OH})_2\text{D}_3$ 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); $1,25(\text{OH})_2\text{D}_3$ 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); oligonucleotides were synthesized by Geneworks (Adelaide, SA, Australia); [α - ^{32}P]dCTP (10 $\mu\text{Ci}/\mu\text{l}$) 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, p*CYP24*-mVDRE1+2-Luc and p*CYP24*-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 PKC ζ , PKC ζ K281 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'-AGCGTGCTCATTTGGC CACTCCAGC-3'; mCCAAT, 5'-CAGCGTGCT **CAGCT**GCCACTCCAGC-3'; wtGC, 5'-CTTCC CACACCCGCCCCCGCGTCCCTC-3'; mGC, 5'-CTTCC CACACCCG**AA**CCCCGCGTCCCT C-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 μ g/ml G418. All plasmids for transfection were prepared by the CsCl/ethidium bromide equilibrium density gradient procedure and quantified by spectrophotometry to ensure experimental consistency. In preparation for transfection, approximately 2×10^5 cells were seeded into 24-well trays in 400 μ l DMEM

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 μ l and mixed with 1.5 μ g DOTAP diluted in 5 μ l 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 μ l 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 (1×10^6 cells) were seeded into one 60 mm Petri dish with 5 ml DMEM medium with 10% fetal calf serum and 200 μ g/ml G418. Once attached, the cells were transfected with the cocktail of 50 μ l DNA mixture (10 μ g promoter construct, 100 ng pEF-IRES-Puro vector kindly provided by Professor John Wallace, University of Adelaide, Adelaide, SA, Australia) and 50 μ l DOTAP mixture (30 μ g DOTAP). The 100 μ l 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 μ g/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 μ g/ml puromycin and grown until confluency was reached. Pooled stably transfected cells (2×10^5) were seeded into 24-well plates in 400 μ l DMEM containing 10% fetal calf serum and 1 μ g/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 (p*CYP24*WT-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). p*CYP24*WT-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), adenylate 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 p*CYP24*WT-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 p*CYP24*WT-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 CGCCCCCG-3') (Fig. 1). Site-directed mutagenesis showed that the EBS did not contribute to basal expression of p*CYP24*WT-Luc, but the GC and CCAAT box sequences were particularly important with inactivation of either site in the constructs p*CYP24*mGC-Luc and p*CYP24*mCCAAT-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 p*CYP24*mVDRE1+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

Figure 1 Transient transfection of wild type and mutant pCYP24-Luc constructs. The wild type and mutant promoter constructs were transiently transfected into HR-12 cells. Following transfection, the cells were treated with calcitonin at 10 nM for 24 h in serum-free RPMI medium. The luciferase activity (LA) represents the mean $\pm$ S.D. for firefly luciferase/ μ g protein content of triplicate samples. The fold induction shown is the ratio of luciferase activity from calcitonin-treated cells to that from untreated cells. Data presented are representative of two independent experiments.

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 ± 0.29 - to 2.53 ± 0.46 - and 2.57 ± 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 ± 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 ± 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 ± 0.67 to 1.64 ± 0.41

and 0.33 ± 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 ± 0.99 to 2.79 ± 0.34 and 1.22 ± 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

Figure 2 The effect of a dominant negative form of NF-Y, NF-YAm29 on basal and calcitonin induction of pCYP24WT-Luc or pCYP24mCCAAT-Luc constructs. HR-12 cells were cotransfected with 200 ng NF-YAm29 together with pCYP24WT-Luc or pCYP24mCCAAT-Luc. After transfection overnight, the cells were treated with 10 nM calcitonin for 24 h in serum-free RPMI medium. The luciferase activity represents the mean±s.d. firefly luciferase activity/μg protein content of triplicate samples. The fold inductions shown in the brackets are the ratio of luciferase activity from calcitonin-treated cells to that from untreated cells. Data are representative of two independent experiments.

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 ± 0.10 -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 $1,25(\text{OH})_2\text{D}_3$ (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 pCYP24WT-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 pCYP24WT-Luc measured after 24 h (the fold induction in the absence and presence of the mutant being 4.67 ± 0.29 and 4.16 ± 0.27 respectively). In a control experiment, the inhibitory effect of this mutant clone was confirmed with induction of pCYP24WT-Luc by $1,25(\text{OH})_2\text{D}_3$ at 100 nM measured after 24 h being lowered by ERK1K71R (from 49.24 ± 3.90 -fold induction 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 PKC ζ 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 pCYP24WT-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 pCYP24WT-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.

Figure 3 Effect of PKA and PKC inhibitors on calcitonin (CT)-mediated induction of CYP24 promoter activity. HR-12 cells were transiently transfected with pCYP24WT-Luc and treated with 10 μM H89 or 100 nM calphostin C for 1.5 h prior to addition of 10 nM CT for a further 24 h incubation. The luciferase activity represents the mean $\pm$ s.d. firefly luciferase/ μg protein content of triplicate samples. Data are representative of two independent experiments.

The atypical PKC isoform PKC ζ 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 pCYP24WT(298)-Luc in HR-12 cells, it was of interest to investigate the possible involvement of PKC ζ . A dominant negative clone of PKC ζ , PKC ζ K281M (Soh *et al.* 1999), when overexpressed at 200 ng did not alter basal expression of pCYP24WT-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 pCYP24WT-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).

Figure 4 The effect of dominant negative mutant PKC ζ K281 M on calcitonin-mediated induction of the *CYP24* promoter. HR-12 cells were transiently transfected with pCYP24WT(298)-Luc or pCYP24mGC(298)-Luc constructs with or without 500 ng PKC ζ K281 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 $\pm$ S.D. of triplicate samples. Data are representative of two independent experiments.

Synergistic effect of calcitonin and 1,25(OH) $_2$ D $_3$ on *CYP24* promoter activity

Since 1,25(OH) $_2$ D $_3$ (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) $_2$ D $_3$ 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-Luc and was dependent on the two VDREs (data not shown). Since 1,25(OH) $_2$ D $_3$ 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-Luc, levels of induction of about 5-fold were observed while in stably transfected cells levels of about 14-fold were seen. Mutagenesis analysis demonstrated that both basal expression and calcitonin induction of the *CYP24* promoter are dependent on a GC box and CCAAT box located at -114/-101 and -62/-51 respectively. Plasmid overexpression studies suggested that Sp1 functions through the GC box while NF-Y acts through the CCAAT box. Data from electrophoretic mobility shift assays have revealed the binding of several proteins from nuclear extracts to both the GC and CCAAT box with supershifting antibodies providing evidence for the binding of Sp1 to the GC box and NF-Y to the CCAAT box, although further work is required to

Figure 5 Synergistic effect of calcitonin and 1,25(OH)₂D₃ on the induction of pCYP24WT-Luc in transiently transfected cells. HR-12 cells were transiently transfected with pCYP24WT-Luc and treated with 10 nM calcitonin or/and 100 nM 1,25(OH)₂D₃ for 24 h. The fold induction shown is the ratio of luciferase activity from calcitonin-treated cells to that from untreated cells representing the mean±S.D. of triplicate samples. Data are representative of three 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.

Figure 6 Proposed events in the induction of the *CYP24* promoter by calcitonin. It is proposed that calcitonin activates PKC and PKA (but not ERK1/2) activities with PKA then acting on Sp1 and NF-Y. Ets-1 does not contribute to basal or calcitonin induction due to the presence of a corepressor complex bound to unliganded VDR/RXR. PKC acting through Ras may stimulate PKC ζ through a PKC–Ras–PI-3K–PDK-1–PKC ζ signaling pathway.

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 ζ as described in other cells (Rodriguez-Viciano *et al.* 1994, 1996, Vanhaesebroeck & Alessi 2000). PKC ζ 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 ζ , PKC ζ K281 M (Soh *et al.* 1999) was employed to investigate whether this isoform plays a role in *CYP24* promoter expression. Blocking endogenous PKC ζ 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 ζ -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 ζ .

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 β and diacylglycerol (DAG) (Chabre *et al.* 1992). PKC could then activate Ras and in turn the Ras–PI-3K–PDK-1–PKC ζ pathway

leading to the phosphorylation and activation of Sp1. However, the data presented are preliminary and implicate only PKC, Ras and PKC ζ 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) $_2$ D $_3$ levels. It is well accepted that 1,25(OH) $_2$ D $_3$ 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) $_2$ D $_3$ 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) $_2$ D $_3$ 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) $_2$ D $_3$ 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) $_2$ D $_3$ (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) $_2$ D $_3$. 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) $_2$ D $_3$. 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) $_2$ D $_3$ and calcitonin correlate with the promoter activities.

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