Short communication

Tissue specific expression and differential regulation by $1\alpha,25$ -dihydroxyvitamin D_3 of the calcium-sensing receptor (CaSR) gene in rat kidney, intestine, and calvaria

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Abstract

The calcium-sensing receptor (CaSR) plays a critical role in calcium (Ca^{2+}) homeostasis, and it exists in Ca^{2+} regulatory tissues such as parathyroid, kidney and intestine. As changes in the quality and quantity of CaSR mRNA may have an effect on sensing of extracellular Ca^{2+} concentration, we analyzed the ontogeny and regulation by $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) of CaSR mRNA expression in the kidney, intestine, and bone. In 6-week-old rats, CaSR mRNA was expressed as a major transcript of 8.5 kb and as minor transcripts of 4.8 and 2.5 kb in the kidney, whereas it appeared as faint transcripts of 8.5, 4.0 and 2.5 kb in the intestine and calvaria. These results showed that CaSR mRNAs were expressed in different structures among these organs. Moreover, the level of CaSR mRNA increased in the kidney from the embryo to the adult. In contrast, the CaSR mRNA level decreased in the intestine during this transition, and the level of it did not change in the calvaria. Moreover, $1\alpha,25(OH)_2D_3$ up-regulated the level of CaSR mRNA in the kidneys in 6-week-old rats. On the other hand, the $1\alpha,25(OH)_2D_3$ did not affect the CaSR mRNA expression in the intestine or calvaria. We concluded that different transcripts of CaSR were expressed in rat kidney, intestine, and calvaria and that the level of CaSR mRNA was different at various developmental stages in the kidney and intestine. Moreover, $1\alpha,25(OH)_2D_3$ regulated the expression of CaSR mRNA only in the kidney.

Abbreviations: AGPC, acid guanidinium thiocyanate-phenol-chloroform; CaSR, calcium-sensing receptor; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $1\alpha,25(OH)_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ; PN, postnatal day; PTH, parathyroid hormone; RT-PCR, reverse transcription-polymerase chain reaction

Introduction

A calcium-sensing receptor (CaSR) was identified in bovine parathyroid gland (Brown et al., 1993) and subsequently, a human homologue of the same receptor was cloned from the parathyroid gland (Garrett et al., 1995). The CaSR, a G-protein-coupled receptor with seven membrane-spanning domains, plays

a critical role in sensing extracellular calcium (Ca^{2+}) concentrations. CaSR mRNA is expressed abundantly in parathyroid gland and kidney (Ruat et al., 1995). The CaSR senses extracellular Ca^{2+} concentrations and responds to its change by regulating parathyroid hormone (PTH) synthesis and secretion by parathyroid cells (Brown et al., 1993). It has been suggested that the effects of extracellular Ca^{2+} on the function of the

renal might be mediated by CaSR (Brown et al., 1993; Pollak et al., 1993). These suggest that CaSR plays a critical role in Ca^{2+} homeostasis. In addition, the expression of the CaSR gene has been demonstrated in bone (Kameda et al., 1998; Yamaguchi et al., 1998), intestine (Gama et al., 1997), thyroid (Garrett et al., 1995), central nervous system (Chattopadhyay et al., 1997; Rogers, 1997), blood monocytes (Yamaguchi et al., 1998), and keratinocytes (Bikle et al., 1996; Oda et al., 1998). The multiple locations suggest that CaSR has a variety of functional roles in the body.

Some reports have described the function of CaSR in Ca^{2+} metabolic organs, such as intestine. The characterization of CaSR in intestinal epithelial cells indicates that CaSR may mediate Ca^{2+} absorption (Gama et al., 1997). It has long been known that $1\alpha,25\text{-dihydroxyvitamin D}_3$ ($1\alpha,25(\text{OH})_2\text{D}_3$) acts to stimulate movement of the divalent cation from the lumen (mucosal side) to the blood (serosal side) of this tissue (Nemere and Norman, 1991). The functions of CaSR have also been described in bone. Isolated osteoclast, and both UMR-106 and SAOS-2 osteosarcoma cell lines were expressed in CaSR protein and mRNA (Yamaguchi et al., 1998; Kameda et al., 1998).

Cytological and histological studies have suggested various roles of CaSR in the body. Furthermore, the variety of CaSR transcripts have been shown in various species. In the bovine species, the major transcript is 5.6 kb in length with a minor transcript of 9.5 kb in the parathyroid gland (Brown et al., 1993), whereas in rat the major transcript is 7.5–8.5 kb with a minor transcript of 5.5–3.8 kb in kidney, brain, thyroid gland, etc. (Ruat et al., 1995; Garrett et al., 1995; Chattopadhyay et al., 1997). In rabbits a single transcript of 6.6 kb was found in the kidney, parathyroid gland, and intestine (Butters et al., 1997), and in chicken a single transcript of 6.2 kb was detected in the parathyroid gland (Diaz et al., 1997). The meaning of the presence of several CaSR transcripts is unclear in each animal.

Two investigations have focused on developmental regulation of CaSR expression. CaSR mRNA and protein increased markedly during the first postnatal week in the kidney (Chattopadhyay et al., 1996). Also, CaSR mRNA and protein levels were expressed at low levels postnatally at 5 days but then increased markedly at 10 days in the hippocampus (Chattopadhyay et al., 1997). However, there is no information available about age-dependent expression of CaSR in either intestine or bone.

Moreover, Ca^{2+} homeostasis is tightly regulated

by the interaction of several hormones such as, PTH, $1\alpha,25(\text{OH})_2\text{D}_3$ and calcitonin, and organ systems such as parathyroid gland, kidney, intestine and bone. $1\alpha,25(\text{OH})_2\text{D}_3$ is known to up-regulate serum Ca^{2+} level through its action on kidney, intestine, and bone (Nemere and Norman, 1991; Matsumoto et al., 1992; Yamato et al., 1993). However, a part of the molecular mechanism for maintenance of Ca^{2+} homeostasis by $1\alpha,25(\text{OH})_2\text{D}_3$ and/or CaSR has not yet been clarified.

To define the role of CaSR in Ca^{2+} metabolic organs, we investigated the regulation of CaSR mRNA expression in the kidney, intestine, and bone during ontogenesis with respect to $1\alpha,25(\text{OH})_2\text{D}_3$ -status. Our results showed that expression of CaSR mRNA was modulated developmentally in the kidney and intestine and that $1\alpha,25(\text{OH})_2\text{D}_3$ only up-regulated the expression of CaSR mRNA in the kidney.

Materials and methods

Animals

Wistar rats from Japan SLC, Inc (Hamamatsu, Shizuoka, Japan) were used for all experiments. The animals used for detection of CaSR mRNA in organs were fetuses taken at the 19th day of pregnancy and rats examined at postnatal day 1, 4, 8, and 42. We picked out male rats in postnatal day 42. Before the experiment, every animal was housed in climate and humidity-controlled, light-cycled rooms and fed standard rat chow with free access to distilled water.

$1\alpha,25(\text{OH})_2\text{D}_3$ treatment

The animals used for this experiment were 6-week-old males. The rats were given intraperitoneal injections of 100 ng of $1\alpha,25(\text{OH})_2\text{D}_3$, dissolved in propylene glycol. Before administration of $1\alpha,25(\text{OH})_2\text{D}_3$, the rats were forced to fast for 24 h in order to prevent any influence from food. In order to determine the time course of the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on CaSR mRNA expression, the rats were killed at 2, 6, 16, and 32 h after a single intraperitoneal injection of 100 ng of $1\alpha,25(\text{OH})_2\text{D}_3$.

RNA preparation and northern blot analysis

The RNA was prepared from the following tissues: 1) whole tissue of cortex and medulla of kidney except blood vessels, 2) jejunum of intestine, 3) calcified fragments of calvaria, after removal of the

periosteum and non-calcified part of the bone marrow by scissors. After RNA was prepared from these tissues, Northern blot analysis was carried out as described below. Total RNAs from kidney and intestine were prepared by the AGPC (guanidinium thiocyanate-phenol-chloroform) method (Chomczynski and Sacchi, 1987). Isolation of RNA from bone was done by the method by Gabor (Nemeth et al., 1989). Poly(A)⁺ RNA was purified with a micro oligo-(dT) cellulose spine column (5 Prime – 3 prime, Inc. Boulder) from total RNA. The RNAs were fractionated on electrophoresed 1% agarose/formaldehyde gels and transferred overnight to nylon membranes. The cDNA probe was prepared from human CaSR cDNA. CaSR cDNA was excised from the vector by EcoRI and NotI digestion, gel-purified, and labeled by random-primed synthesis with ³²P-dCTP to a specific activity of 1×10^6 dpm μ g (Feinberg and Vogelstein, 1983; Clark et al., 1987). The nylon membranes with transferred RNA were hybridized in a solution consisting of 0.5 M NaHPO₄, (pH 7.2), 1% BSA, 1 mM EDTA, and 7% SDS with a CaSR probe at a concentration of 1.9×10^9 dpm μ g. Blots were washed sequentially three times for 20-min periods, in the following solutions: first with $2 \times$ SSPE, 0.03% NaPPi, 0.1% SDS solution, second with $1 \times$ SSPE, 0.03% NaPPi, 0.1% SDS solution, and third with $0.1 \times$ SSPE, 0.03% NaPPi, 1% SDS solution. Membranes were exposed to X-ray film for 4 days at -80°C . An equivalent blot was probed with ³²P-labeled cDNA probes for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a load control. The message size was estimated by comparison with the mobility of molecular weight markers (RNA ladder, 0.2–10 kb, Novagen, USA). RNA loading film was scanned with a SHARP JX-330M scanner and quantified by NIH Image.

Results and discussion

Tissue specific expression of CaSR mRNA

Northern blot analysis was performed on poly(A)⁺ RNA prepared from 6-week-old (42 days) male rats with hCaSR cDNA as a probe. Northern blot analysis showed the expression of CaSR mRNA in the kidney, intestine, and calvaria. This probe demonstrated several sizes of CaSR mRNAs (including 8.5, 4.8, 4.0, and 2.5 kb) in poly(A)⁺ RNA from rats (Figure 1). CaSR mRNAs in the kidney were identified as a major transcript of 8.5 kb and minor transcripts of 4.8,

Figure 1. Northern blot analysis of CaSR transcripts in rat kidney, intestine, and calvaria. Northern blot analysis was performed on poly(A)⁺ RNA prepared from 6-week-old rats (postnatal day 42). Poly(A)⁺ RNA preparations from kidney (3 μ g), intestine (6 μ g), and calvaria (6 μ g) were used. CaSR mRNAs in the kidney were 8.5, 4.8, 4.0, and 2.5-kb transcripts, with the major transcript being 8.5 kb. CaSR mRNAs in the intestine and calvaria were 8.5-kb, 4.0-kb, and 2.5-kb transcripts. Molecular sizes (kb) are shown. The experiment was done four times, and similar results were obtained each time.

4.0, and 2.5 kb. The expression level of total CaSR mRNAs was lower in the intestine and calvaria than in the kidney. The 8.5-kb, 4.0-kb, and 2.5-kb transcripts were observed in the intestine and calvaria.

This is the first report that clearly demonstrates a difference in transcript length of CaSR mRNA in the kidney, intestine, and calvaria in rats. Ruat et al. had suggested that the thyroid, including parathyroid tissue, had a substantial amount of expression with bands at 8.5 and 3.8 kb for CaSR transcripts, whereas kidney had 5.5-kb band and the 8.5-kb band (Ruat et al., 1995). These results are consistent with our data. Their 5.5-kb transcript corresponds with our 4.8-kb transcript in the kidney, and their 3.8-kb transcript corresponds with our 4.0-kb transcript in other tissues. These variations of CaSR mRNA structure may be due to processing variants derived from the CaSR gene, but it is not currently known whether these transcripts are splice variants, or reflect the use of alternative promoter or polyadenylation sites within the same gene. The different forms of mRNA may differ in their stability or the efficiency with which they are translated. One study utilizing RT-PCR analysis of CaSR mRNA in the thyroid gland indicated that there was no alternative splicing within the coding region (Rogers et

al., 1995). On the other hand, an alternatively spliced form that lacked exon 5, and encoded a portion of the extracellular domain of CaSR mRNA was observed in normal keratinocytes (Oda et al., 1998). Although we are not able to clarify the mechanism of expression of several transcripts from CaSR gene, our results indicate tissue/cell-specific roles for CaSR.

Developmental regulation of CaSR mRNA expression

The increase in CaSR mRNA was age dependent, from embryonic day (E) 19 to postnatal day (PN) 42 in the kidney (Figure 2A). Moreover, the 2.5-kb transcript of CaSR was strongly expressed in the embryonic kidney. On the other hand, we found an age-dependent decrease in CaSR mRNA in the intestine, and the level of its transcript decreased until postnatal day 4, when it was nearly at the adult level of expression (Figure 2B). However, the level of CaSR mRNA in the calvaria showed no significant change in accordance with the developmental stage (Figure 2C).

Then we showed that the expression level of CaSR mRNA in the intestine decreased according to the developmental stage, and that the expression level of CaSR mRNA in the calvaria remained the same throughout ontogenesis. Moreover, our results taken together with those of Chattopadhyay et al. showed that expression level of CaSR mRNA in the kidney increased dependent on the developmental stage (Chattopadhyay et al., 1996). These dramatic up- and down-regulations of CaSR mRNA levels by ontogenesis during the suckling stage suggest that CaSR has a specific role in each developmental stage. These results may be related to the way by which Ca^{2+} intake occurs, such as from maternal blood or milk or from chow. Studies on Ca^{2+} metabolism in homozygous CaSR knockout mice may be instructive in this regard (Ho et al., 1995). These animals died between days 3 and 30 of life. These reports and ours suggest that the CaSR plays an important role in Ca^{2+} metabolism during the suckling stage.

Tissue specific regulation of CaSR mRNA expression by $1\alpha,25(\text{OH})_2\text{D}_3$

We next examined the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ treatment on gene expression of CaSR in the kidney, intestine, and calvaria of rats. The data for each organs is shown in Figure 3. The results of these experiments show the level of total CaSR mRNAs in various tissues resulting from treatment with $1\alpha,25(\text{OH})_2\text{D}_3$, as

Figure 2. Developmental expression of CaSR transcripts in rat kidney (A), intestine (B), and calvaria (C). Northern blot analysis for CaSR mRNA. The 8.5-kb and 4.8-kb transcripts increased from embryonic day 19 (E19) to postnatal day 42 (PN42) (A). CaSR mRNA expression in the intestine was strong on embryonic day 19 (E19), and then decreased on postnatal days 1 (PN1) and 4 (PN4) age dependently (B). The expression level of CaSR mRNA was constant throughout development in the calvaria (C). Poly(A)⁺ RNA (3 μg) was used in each experiments. GAPDH mRNA expression was used as the positive control. Data are representative of those obtained in four independent experiments. Molecular sizes (kb) are shown.

Figure 3. Effect of $1,25(\text{OH})_2\text{D}_3$ treatment on the level of CaSR mRNA in rat kidney (A), intestine (B), and calvaria (C). Northern blot analysis for CaSR mRNA performed on poly(A)⁺ RNA from the kidney, intestine, and calvaria of 6-week-old (PN42) rats treated with $1,25(\text{OH})_2\text{D}_3$, compared with untreated controls. Animals were injected intraperitoneally with 100 ng of $1,25(\text{OH})_2\text{D}_3$ in 100 μl of propylene glycol ($1,25(\text{OH})_2\text{D}_3$) or with 100 μl of propylene glycol alone (control), and killed at various times. Results were normalized to GAPDH mRNA expression. Analyse of the CaSR mRNA-to-GAPDH mRNA ratio performed on 4.8-kb transcript in kidney and 4.0-kb transcript in intestine and calvaria. The values are expressed as means $\pm$ S.D. of samples from at least four different rats. * $p < 0.05$.

revealed by Northern blotting. The CaSR mRNA-to-GAPDH mRNA ratio moderately increased by 30.8% in the kidney ($n = 3$, $p < 0.05$, three independent experiments), within 2 h after giving $1,25(\text{OH})_2\text{D}_3$ (Figure 3A). In contrast, $1,25(\text{OH})_2\text{D}_3$ treatment did not show any effective regulation on the expression of CaSR mRNAs in the calvaria or intestine (Figures 3B and C). Analyse of the CaSR mRNA-to-GAPDH mRNA ratio performed at 4.8-kb transcripts in the kidney, 4.0-kb transcripts in the intestine and calvaria.

As $1,25(\text{OH})_2\text{D}_3$ up-regulated the level of CaSR mRNA in the kidney within 2 h after the treatment, $1,25(\text{OH})_2\text{D}_3$ may regulate the expression of CaSR gene at the transcriptional level. We presume that mechanism for altering the set point for extracellular Ca^{2+} would be an action on the expression and/or functional properties of the CaSR. On the other hand, as $1,25(\text{OH})_2\text{D}_3$ did not affect the CaSR mRNA level in either intestine or calvaria, these results show that the regulation of CaSR mRNA expression by $1,25(\text{OH})_2\text{D}_3$ occurs in a tissue specific manner. There are two reports about whether $1,25(\text{OH})_2\text{D}_3$ regulates the level of CaSR mRNA in the kidney and parathyroid. One *in vivo* study indicated that treatment of rats with $1,25(\text{OH})_2\text{D}_3$ up-regulated the level of CaSR mRNA in the kidney and parathyroid (Brown et al., 1996), whereas the other showed no effect (Rogers et al., 1995). Brown et al. had suggested that the rapid return of CaSR mRNA to pretreatment levels is a relatively short half-life for this transcript. There has been no report on this point with respect to intestine or bone. Our results, together with those of Brown et al show that $1,25(\text{OH})_2\text{D}_3$ regulates the expression of CaSR mRNA in the kidney, even though different of experimental procedures were used.

In summary, we have demonstrated evidence for the expression of different transcripts of the CaSR gene from several tissues, and tissue-specific regulation of CaSR mRNA expression by ontogenesis and $1,25(\text{OH})_2\text{D}_3$. These results suggest that the CaSR has multiple functions in Ca^{2+} signaling in rats.

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