



Calcium-Sensing Receptor Gene: Regulation of Expression

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The human calcium-sensing receptor gene (*CASR*) has 8 exons, and localizes to chromosome 3q. Exons 1A and 1B encode alternative 5'-untranslated regions (UTRs) that splice to exon 2 encoding the AUG initiation codon. Exons 2–7 encode the CaSR protein of 1078 amino acids. Promoter P1 has TATA and CCAAT boxes upstream of exon 1A, and promoter P2 has Sp1/3 motifs at the start site of exon 1B. Exon 1A transcripts from the P1 promoter are reduced in parathyroid tumors and colon carcinomas. Studies of colon carcinomas and neuroblastomas have emphasized the importance of epigenetic changes—promoter methylation of the GC-rich P2 promoter, histone acetylation—as well as involvement of microRNAs in bringing about *CASR* gene silencing and reduced CaSR expression. Functional cis-elements in the *CASR* promoters responsive to 1,25-dihydroxyvitamin D [1,25(OH)₂D], proinflammatory cytokines, and the transcription factor glial cells missing-2 (GCM2) have been characterized. Reduced levels of CaSR and reduced responsiveness to active vitamin D in parathyroid neoplasia and colon carcinoma may blunt the “tumor suppressor” activity of the CaSR. The hypocalcemia of critically ill patients with burn injury or sepsis is associated with *CASR* gene upregulation by TNF-alpha and IL-1beta via kappaB elements, and by IL-6 via Stat1/3 and Sp1/3 elements in the *CASR* gene promoters, respectively. The *CASR* is transactivated by GCM2—the expression of which is essential for parathyroid gland development. Hyperactive forms of GCM2 may contribute to later parathyroid hyperactivity or tumorigenesis. The expression of the CaSR—the calciostat—is regulated physiologically and pathophysiologically at the gene level.

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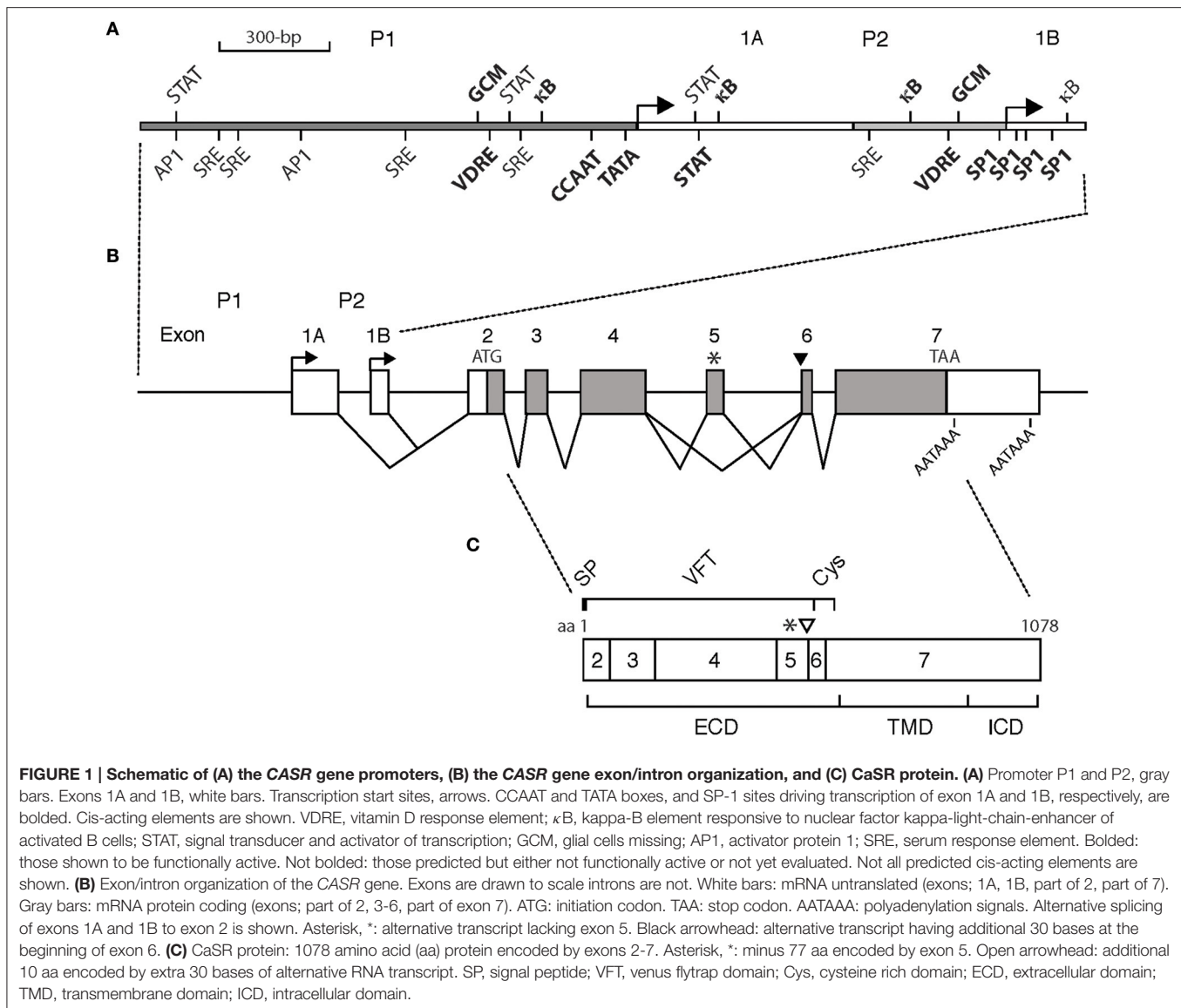
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THE CASR GENE

The single-copy *CASR* gene that maps to human 3q13.3-21 encodes a Class C G protein-coupled receptor family member (Pollak et al., 1993; Janicic et al., 1995). The T-cell antigen *CD86* gene lies upstream and the cysteine protease inhibitor *CSTA* gene downstream of the *CASR* gene and all are transcribed in the same 5' to 3' direction.

The *CASR* gene has eight exons and spans ~100-kb (Yun et al., 2007) (**Figure 1**). Exons 2 to 7 encode the CaSR protein of 1078 aa (GenBank #U20759). Two different polyadenylation signal sequences within exon 7 may be used, to generate either a short (177-nucleotide) or a long (1304-nucleotide) 3'-untranslated region (UTR) (Aida et al., 1995; Garrett et al., 1995). Exon 2 encodes 242 nucleotides of the 5'-UTR upstream of the ATG translation initiation codon. Exons 1A and



1B encode alternative 5'-UTRs that splice to a common segment encoded by exon 2 (Garrett et al., 1995; Chikatsu et al., 2000).

This organization of exons, conserved around primary protein domains, is first seen in aquatic vertebrates (Naito et al., 1998). With diversification from the teleost fishes to tetrapods, mammals, and primates, the evolutionary changes have been greatest in the 5' and 3' domains. While overall exonic structure is preserved a striking increase in intron size has occurred from teleost fish to higher species (Loretz, 2008). Linkage disequilibrium analysis across the human gene shows a central haplotype block extending from exon 2 to 7 that is distinct from separate haplotype blocks for genetic variants in the 5' and 3' flanking regions (Yun et al., 2007).

Thus, the mouse, rat and human genes are organized in a similar manner. Both rodent genes comprise at least 7 exons and the translational start site is in exon 2. Alignment of expressed sequence tags (ESTs) and cloned rodent cDNAs reveal that, just

like the human CASR gene, mouse and rat CASR genes have at least 2 distinct 5'UTRs (Exons 1A and 1B), suggesting the presence of at least 2 promoters. The mouse and rat CASR genes share >85% nucleotide identity (exons and introns) and 40% with the human CASR. The VDREs and κ B elements (see below) that we have characterized in our studies of the human CASR gene promoters are conserved in the rodent *Casr* promoters.

Alternative Transcripts

The CASR gene is highly expressed in the parathyroid gland and renal tubule (Brown et al., 1993). However, the gene is widely expressed at lower levels in other tissues, for example in liver (Canaff et al., 2001), bone (Goltzman and Hendy, 2015) and in lung, breast, placenta, vasculature and gut (Brennan et al., 2013). Several alternative transcripts have been identified, raising the question of their function and regulation of their expression.

Coexistence of transcripts encoding the same 1078 amino acid protein in human parathyroid but different 5' UTRs suggested alternative splicing (Garrett et al., 1995). Moreover, the transcripts may have either a short or long 3' UTR. One low abundance transcript encodes an additional 10 amino acid stretch inserted after amino acid 536 in the exodomain (GenBank #U20760) but does not alter CaSR function (Garrett et al., 1995). The predominant transcript is 5.4-kb in length, while less abundant transcripts of 10, 4.8, and 4.2-kb are also found (Garrett et al., 1995). In human kidney, the 5.4-kb transcript is similarly predominant with the less abundant 10-kb transcript also present (Aida et al., 1995).

The human CASR gene has two promoters driving transcription of alternative 5' UTR exons (1A and 1B) (Chikatsu et al., 2000). By northern blot analysis of human parathyroid adenomas and normal glands, the 5.4 and 10-kb transcripts (see above) appeared to be exclusive to exon 1A use, while the 4.2-kb transcripts are derived from either 1A or 1B (Chikatsu et al., 2000). Real-time PCR (qPCR) analysis of human parathyroid cells revealed that exon 1B-transcripts were much more highly expressed than exon 1A-containing transcripts (Mizobuchi et al., 2009). Transcripts of the 5.4 and 4.2-kb size derive from use of the two alternative polyadenylation sites in the 3' UTR tract (Chikatsu et al., 2000).

An exon 3-deleted CASR transcript has been reported in thyroid TT cells (Freichel et al., 1996), in placental cytotrophoblast (Bradbury et al., 1998), and in parathyroid, thyroid, and kidney (D'Souza-Li et al., 2001). Fusion of exon 2 to exon 4 results in a truncated protein that is poorly expressed and not trafficked to the cell surface.

In human keratinocytes, an alternatively spliced transcript lacking exon 5 encodes a variant CaSR with a 77-amino acid in-frame deletion in the exodomain (Oda et al., 1998). This variant exerts a dominant negative effect on the full-length protein making it less responsive to calcium. In addition, the relative amounts of full-length vs. alternatively spliced transcript decrease during keratinocyte differentiation (Oda et al., 1998).

Consideration of alternatively spliced forms are of importance in phenotype evaluation of *Casr* mice knocked out by deletion of exon 5 (Ho et al., 1995). In the growth plate (Rodriguez et al., 2005), skin (Oda et al., 2000) and kidney (Oda et al., 2000) of the knockout mice, the *Casr* message lacking exon 5 is upregulated and compensates for the absence of the full-length counterpart in bone and cartilage (Rodriguez et al., 2005). Studies of *Casr* knockouts in which both full-length and exon 5 transcripts are deleted have suggested that the exon 5-deletion model might be hypomorphic with respect to CaSR actions in the skeleton (Chang et al., 2008).

Transcriptional Control of the CASR Gene

Regulated CASR gene expression is important in growth and development (Riccardi et al., 2013), and in normal adult physiology (Brown, 2013) and in disease pathogenesis (Hannan and Thakker, 2013). Some of the factors and mechanisms involved in transactivation of the CASR gene have been identified (Hendy et al., 2013).

Human CASR transcription is driven by either promoter P1, with a TATA box at nucleotide -26 and a CCAAT box at -110 relative to the start site, or P2 with an Sp1/3 site at the transcriptional start site (Figure 1). The rat and mouse *Casr* genes have a similar organization. Both promoters drive significant levels of basal activity, with promoter P2 being 2.5-fold more active than P1 in most cell types examined as assessed by transfected promoter-reporter analysis (Canaff and Hendy, 2002). Also nuclear run-on assays that directly measure transcripts suggest greater exon 1B relative to exon 1A transcripts in human thyroid C-cells and renal proximal tubular cells. The presence of multiple promoters provides the potential for tissue-specific and/or developmental/temporal-specific regulated expression from one promoter vs. responsiveness to hormonal or nutritional stimuli from the other. Evidence for this has yet to be fully realized for the CASR gene. In fact, from studies done so far the opposite seems to be the case in that both promoters respond to active vitamin D, cytokines, and the parathyroid cell-specific regulator, GCM2 (see below). However, pathophysiological differences in usage of the CASR gene promoters have been suggested.

Chikatsu et al. (2000) showed the expression of exon 1A transcripts driven by the upstream promoter (P1), was decreased in adenomas, whereas expression of exon 1B transcripts driven by the (normally stronger) promoter P2 was unchanged. These results suggested that P1 activity was reduced in parathyroid adenomas and is qualitatively in agreement with several studies showing a decrease in CaSR mRNA and protein expression in parathyroid adenomas relative to normal glands (Kifor et al., 1996; Farnebo et al., 1997; Gogusev et al., 1997; Cetani et al., 2000; Corbetta et al., 2000). However, other mechanisms yet to be identified are likely to contribute to the greater relative decreases in mRNA and protein expression that are not accounted for quantitatively by the ~50% decrease of the apparently weaker P1 promoter. In a study of colorectal tumors, Kállay et al. (2003) found exon 1A transcript expression to be greater in the adjacent "normal" mucosa of colon cancer patients than in the tumor itself, and a highly significant decrease of exon 1A expression was found during progression from well-differentiated to poorly differentiated cancers whereas exon 1B transcript expression was not significantly altered. More recent studies have found hypermethylation of promoter P2 that drives the exon 1B transcripts (Fetahu et al., 2014a) and therefore reduced exon 1B transcript levels would be predicted. Further studies will be needed to resolve this apparent discrepancy.

Vitamin D

Active vitamin D increases CaSR expression and this has been documented in several studies in rodents. Parathyroid CaSR mRNA was reduced by 40% in vitamin D-depleted relative to replete rats, and 1,25(OH)₂D₃ administration to vitamin D-replete rats enhanced parathyroid and kidney CaSR mRNA levels further (Brown et al., 1996). In another study administration of 1,25(OH)₂D₃ to rats upregulated renal CaSR mRNA levels in a dose- and time-dependent manner (Yao et al., 2005). Kidney CaSR expression (microarray analysis) was downregulated in VDR null mice relative to their wildtype littermates (Li et al.,

2003) and likewise (ribonuclease protection assay) for *Cyp27*^{-/-} mice lacking the 25-hydroxyvitamin D-1 α -hydroxylase enzyme (Canaff et al., 2009a). Injection of 1,25(OH)₂D₃ in either normal mice or the *Cyp27b1*^{-/-} mice caused upregulation of CaSR expression in parathyroid/thyroid and kidney (Li et al., 2003; Canaff et al., 2009a). Nuclear run-on assays that measure RNA transcripts showed that upregulation in thyroid and kidney cells occurred via gene transcription (Canaff and Hendy, 2002). Functional vitamin D response elements (VDREs) are present in both promoters, P1 and P2, of the human *CASR* gene (Canaff and Hendy, 2002) and are conserved in the rodent (Hendy et al., 2013). The VDREs of the *CASR* are typical in that they consist of two 6-bp half-sites separated by 3-bp that are bound by the vitamin D receptor (VDR)-retinoic acid X receptor (RXR) dimer. However, the VDREs are atypical in that the orientation of the half-sites is inverted to that which is normally found. VDREs of this type are found in the 25-hydroxyvitamin D-24-hydroxylase (*CYP24A1*) gene.

In the parathyroid gland, the 1,25(OH)₂D-upregulated CaSR makes the gland more responsive to extracellular Ca²⁺ and PTH secretion is reduced. The negative action of extracellular Ca²⁺ on PTH synthesis and secretion and parathyroid cell proliferation is reinforced by the active vitamin D metabolite. Impaired extracellular Ca²⁺-sensing that drives increased parathyroid cell proliferation may contribute to parathyroid neoplasia. Somatic *CASR* mutations are rare, but parathyroid glands of patients with primary or severe uremic secondary hyperparathyroidism often have reduced CaSR expression (Kifor et al., 1996; Cetani et al., 2000; Corbetta et al., 2000). Thus, reduction in components of the vitamin D system, active vitamin D ligand either circulating levels or produced by parathyroid intracrine action, and/or target VDR levels, that normally decrease PTH synthesis and secretion, could play an additional role by negating the normal inhibitory effects of extracellular Ca²⁺ on PTH via decreased CaSR expression.

The activated kidney CaSR that can act independently of PTH to directly determine the circulating Ca²⁺ concentration (Loupy et al., 2012) inhibits the paracellular uptake of cations in the cortical thick ascending limb of the distal nephron and promotes hypercalciuria. Autosomal dominant hypocalcemia type 1 (ADH1) patients are heterozygous for a gain-of-function mutant *CASR*. Treatment of ADH1 patients with active vitamin D upregulates the oversensitive renal CaSR stimulating Ca²⁺ excretion provoking nephrocalcinosis, nephrolithiasis, and potential renal damage (Pearce et al., 1996; Lienhardt et al., 2001). For such patients a better treatment option is vitamin D itself whereby the amount 1,25(OH)₂D formed is limited by product inhibition of the 25-hydroxyvitamin D-1 α -hydroxylase enzyme in the proximal tubule (Obermannova et al., 2016). In some genetic forms of hypercalciuria, altered regulation of CaSR expression by vitamin D metabolites may be a critical factor contributing to stone formation. In a genetic stone-forming rat model of hypercalciuric nephrolithiasis, nephron VDR levels are elevated, CaSR levels are increased and calcium reabsorption reduced (Yao et al., 2005; Bai and Favus, 2006).

Alterations of CaSR expression have been implicated not only in hyperparathyroidism but also other neoplasms. The CaSR is expressed in human colon epithelium and regulates cell

proliferation and differentiation. Cells of the colon crypt acquire CaSR expression as they differentiate and migrate toward the apex of the crypt (Chakrabarty et al., 2005). CaSR expression is weak or absent in colon carcinomas and is inversely correlated with differentiation status. Extracellular Ca²⁺ and 1,25(OH)₂D upregulate *CASR* transcription and cyclin-dependent kinase inhibitor expression in the colon and Ca²⁺ and 1,25(OH)₂D may exert their chemopreventative actions with respect to colon cancer, in part, through the CaSR (Chakrabarty et al., 2005). Fetahu et al. (2014b) demonstrated the upregulation of CaSR expression by 1,25(OH)₂D₃ in colon cancer cells. This would be consistent with the active vitamin D metabolite exerting its antiproliferative, prodifferentiating effects in part by inducing expression of the tumor suppressor, CaSR.

Vascular calcification occurs during aging and pathologically in atherosclerosis and chronic kidney disease. There is an inverse relationship between vascular calcification and expression of the CaSR (Alam et al., 2009) and maintenance of its expression can protect against calcification (Molostvov et al., 2015). Treatment of vascular smooth muscle cells with active vitamin D increases CaSR mRNA and protein levels (Mary et al., 2015). This would be consistent with 1,25(OH)₂D transactivation of the *CASR* gene via its VDREs (Canaff and Hendy, 2002). Treatment with cinacalcet that enhances sensitivity of the CaSR to Ca²⁺ promotes CaSR expression at the cell surface while overall CaSR expression is not altered (Hénaut et al., 2014). This would be consistent with ligand induced trafficking of the CaSR to the cell surface (Breitwieser, 2013) and Ca²⁺ within the normal range and higher having no marked effect on *CASR* gene expression (see below for further detail of potential regulation of *CASR* by extracellular Ca²⁺).

Calcium

In addition to 1,25(OH)₂D, a potential regulator of *CASR* gene expression is extracellular Ca²⁺ itself. Initial studies did not find an effect of extracellular Ca²⁺ on parathyroid gland or whole kidney CaSR mRNA in the rat *in vivo* (Rogers et al., 1995; Brown et al., 1996). A lack of effect on *CASR* expression by circulating Ca²⁺ is perhaps not unexpected in tissues such as parathyroid gland or kidney, where the CaSR plays a critical role in Ca²⁺ homeostasis as a calciostat to sense small changes in extracellular Ca²⁺ concentration. Even slight alterations in the extracellular Ca²⁺ set-point (the extracellular Ca²⁺ concentration at which PTH secretion from the parathyroid gland or calcium reabsorption across the kidney tubule is half-maximal) brought about by changes in CaSR synthesis could disturb overall calcium homeostasis.

CaSR expression did not change in rats having altered circulating calcium levels as a result of infusion of CaCl₂ or fed vitamin D-deficient diets supplemented with Ca²⁺ (Rogers et al., 1995; Brown et al., 1996). In these two studies the extracellular calcium levels ranged from 0.7 to 1.9 mM. However, potentially, lower or higher Ca²⁺ concentrations could regulate CaSR expression and perhaps in a tissue-specific manner.

CASR gene transcription was modulated by extracellular Ca²⁺ in studies of transfected *CASR* promoter-reporter constructs in human kidney proximal tubule cells and mouse distal convoluted tubule cells (Canaff, 2004). In both cell types, when

the concentration of Ca^{2+} in the media was varied from 1 to 5 mM, there was only a slight increase in P1 and P2 promoter activity. However, there was markedly reduced transcriptional activity of P1 (by 50%) and P2 (by 30%) in both proximal and distal convoluted tubule cell types when cultured in a very low (0.25 mM) Ca^{2+} concentration. Therefore, from normal to high Ca^{2+} there was little regulation but from normal to low calcium there was significant regulation. The results from these *in vitro* experiments would be consistent with the lack of obvious effect of circulating Ca^{2+} in the *in vivo* studies referred to above (Rogers et al., 1995; Brown et al., 1996). In other cells—differentiated human colon carcinoma cells—upregulation of the CaSR occurred when the Ca^{2+} concentration of the culture medium was increased from very low to normal (Chakrabarty et al., 2005). Stimulatory effects of Ca^{2+} and $1,25(\text{OH})_2\text{D}$ were additive for promoter P2 but not for promoter P1 (Chakrabarty et al., 2005).

What would be the significance in the kidney of a reduced CaSR expression at low extracellular Ca^{2+} concentration? A decrease of CaSR expression in the proximal tubule would decrease the effect of calcium on the 25-hydroxyvitamin D-1 α -hydroxylase activity i.e., the negative impact of Ca^{2+} in decreasing enzyme activity would be minimized. In the ascending limb and distal convoluted tubule, the combination of a reduced CaSR expression and a low extracellular Ca^{2+} would favor maximal Ca^{2+} reabsorption from the tubule lumen and allow PTH to stimulate Ca^{2+} reabsorption without being antagonized by the CaSR.

The precise pathways by which extracellular Ca^{2+} might transactivate the *CASR* gene are not known. If the extracellular Ca^{2+} effect is mediated via the CaSR then a transcription factor activated by mitogen activated protein kinase (MAPK) (e.g., Elk1) could be involved. In addition, the involvement of transcription factors in the reduced expression of the CaSR in neoplasia of parathyroid and colon, for example, are not known. In an *in vivo* murine model of primary hyperparathyroidism in which cyclin D1 overexpression is targeted to the parathyroid, expression of the CaSR in the gland is markedly reduced and correlates with the severity of the hyperparathyroidism (Kawata et al., 2005). Treatment of the mice with the allosteric CaSR activator cinacalcet to mimic high levels of extracellular Ca^{2+} suppressed serum PTH and Ca^{2+} and parathyroid cell proliferation but had no effect on the levels of parathyroid CaSR mRNA (Imanishi et al., 2011). This reinforces the view that normal or higher extracellular Ca^{2+} concentrations do not significantly modulate parathyroid CaSR expression levels.

Proinflammatory Cytokines

Evidence has accumulated that the increased levels of circulating cytokines such as IL-1 β and IL-6 occurring in conditions of inflammation could provoke altered systemic Ca^{2+} homeostasis by modulating the setting of the calcostat—the expression of the CaSR. In critically ill patients such as those with sepsis and major burn injury hypocalcemia is common (Zaloga, 1992; Zivin et al., 2001; Steele et al., 2013) and in non-acutely ill patients undergoing surgery (Lepage et al., 1999). Serum IL-1 β and IL-6 levels increase within hours of severe burns and infection

(Kowal-Vern et al., 1994; Klein et al., 1995; Caldwell et al., 1997), and are inversely related to the falls in serum Ca^{2+} concentration (Lind et al., 2000) that may correlate with a poor prognosis (Guo et al., 1990; Nijsten et al., 1991; Schlüter et al., 1991; Ohzato et al., 1993; Yamada et al., 1996; Remick et al., 2002). Several factors such as decreased secretion of PTH and/or resistance to PTH action in kidney and bone may contribute to the hypocalcemia (Katz et al., 1992; Klein et al., 1997). In addition, the metabolism and action of vitamin D can be impaired. Calcitonin precursors are increased in the circulation of critically ill patients with sepsis (Lind et al., 2000; Müller et al., 2000; Becker et al., 2004, 2010). Certainly altered PTH secretion and action and vitamin D metabolism will be most important, whereas calcitonin plays a minor role in Ca^{2+} homeostatic control in human.

In patients with rheumatoid arthritis impaired secretion of PTH is inversely related to the inflammatory activity (Ekenstam et al., 1990). PTH secretion from bovine parathyroid cells was suppressed by IL-6 *in vitro* (Carlstedt et al., 1999) and the same effect was demonstrated for clinically relevant doses of IL-1 β acting specifically via the IL-1 receptor in bovine parathyroid tissue slices *in vitro* with the added observation of upregulation of CaSR mRNA levels (Nielsen et al., 1997). Parathyroid CaSR mRNA levels were upregulated in an *in vivo* sheep model of burn injury in which increased circulating cytokine levels would occur (Murphey et al., 2000). Some proinflammatory cytokines can stimulate (rather than inhibit) the release of PTH. The IL-8B receptor (CXCR2) was identified in the bovine parathyroid gland (Angeletti et al., 1998). Cultured bovine parathyroid cells responded to IL-8 with increases in PTH mRNA levels and PTH secretion, although any role of altered CaSR expression in these effects is as yet unknown.

Dysregulated PTH release and altered circulating Ca^{2+} levels occurs in septic horses (Toribio et al., 2001; Hurcombe et al., 2009). Equine parathyroid cells in culture responded to elevations in extracellular Ca^{2+} (0.8–2 mM) with decreased PTH mRNA while CaSR mRNA expression was unaltered consistent with other studies (see above) showing lack of an effect of extracellular Ca^{2+} on CaSR expression within this concentration range. IL-1 β acting via the IL-1 receptor decreased both PTH secretion and increased CaSR mRNA expression (Toribio et al., 2003). In rats *in vivo* serum PTH and $1,25(\text{OH})_2\text{D}$ decreased significantly 12 h after intraperitoneal (ip) injection of IL-1 β , followed by significantly decreased levels of serum Ca^{2+} at 15 h (Canaff and Hendy, 2005). In the same model, decreases in serum PTH and $1,25(\text{OH})_2\text{D}_3$ and calcium occurred after ip injection of IL-6 (Canaff et al., 2008b).

The CaSR in part mediates the antiproliferative and prodifferentiation actions of Ca^{2+} in colonocytes and can be considered as a tumor suppressor in the colon. Fetahu et al. (2014b) showed that in two differentiated colonocyte cell lines CaSR mRNA and protein levels increased in response to the cytokines, TNF α , IL-1 β , and IL-6. The robust increase of CaSR expression could represent a defense against inflammation similar to what had been shown in murine macrophages, in which lipopolysaccharide-induced TNF α release upregulated CaSR leading to negative feedback inhibition of synthesis of the cytokine (Kelly et al., 2011).

Proinflammatory cytokines, like IL-1 β and TNF- α , work through NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells). NF- κ B exists in the cytoplasm of cells in an inactive form bound to an inhibitor, I κ B. Upon receipt of a signal generated by activation of cytokine receptors, I κ B is phosphorylated, and NF- κ B is released from I κ B and translocates to the nucleus to upregulate specific gene transcription via specific κ B response elements. NF- κ B is responsible for the expression of many immune and inflammatory response (and other) proteins (Ghosh et al., 1998; Baldwin, 2001).

In the rat *in vivo* IL-1 β upregulates parathyroid, thyroid, and kidney CASR mRNA and protein levels and stimulates endogenous CASR gene transcription in human thyroid and kidney cell lines (Canaff and Hendy, 2005). In addition, we showed that IL-1 β and TNF- α upregulate CASR gene transcription via NF- κ B with functional κ B response elements being present in both promoters of the CASR gene (Canaff and Hendy, 2005).

At the cell surface, IL-6 binds the IL-6 receptor (IL-6R) consisting of an IL-6 binding α chain (gp80) and the gp130 signal transducer that is shared among the IL-6-related cytokine subfamily members. IL-6 binding to its receptor activates Janus kinase (JAK) family members that phosphorylate and activate signal transducers and activators of transcription (STAT) family members. The STATs dimerize, translocate to the nucleus and bind specific gene STAT-response elements activating transcription (Horvath, 2000; Levy and Darnell, 2002). STAT-1 and STAT-3 are responsible for IL-6 signaling. The JAKs may also couple to the MAPK pathway to modify the activity of transcription factors including STATs and others (Heinrich et al., 2003).

Intraperitoneal injection of IL-6 to rats *in vivo* caused decreases in serum PTH, 1,25(OH) $_2$ D, and Ca $^{2+}$ that were maintained over 24 h (Canaff et al., 2008b). Parathyroid, thyroid and kidney CaSR mRNA and protein levels were upregulated. IL-6 increased CASR gene transcription in human thyroid C-cell and kidney proximal tubule cells *in vitro*. CASR gene P1 and P2 promoter-driven transcripts were upregulated. Activation of the MAPK pathway contributed modestly to basal activity of both P1 and P2 promoters, but it was not involved in a major way in the IL-6 induction of either of them (Canaff et al., 2008b). For promoter P1 a STAT1/3 element downstream of the transcriptional start site accounted for the IL-6 induction. For promoter P2 that has no STAT elements, IL-6 rapidly promotes a complex containing both Sp1/3 and STAT1/3 on GC-rich elements that are clustered at the transcription start site.

The IL-6 administration to rats resulted in a more rapid decrease in serum PTH, 1,25(OH) $_2$ D and Ca $^{2+}$ levels than that occurring after IL-1 β administration (Canaff and Hendy, 2005; Canaff et al., 2008b). The quicker response to IL-6 may relate to this cytokine's ability to induce rapid changes in mRNA translation and protein synthesis via the eukaryotic initiation factor-4E, as has been shown in sensory neurons, for example (Melemedjian et al., 2010). While IL-6 clearly regulates CASR gene transcription, uncovering any contribution of the cytokine at the post-transcriptional level with respect to the resetting of the calcostat will require further study.

Other Considerations

At sites of injury or infection the inflammatory response increases circulating proinflammatory cytokines leading to increased bone resorption. This occurs by enhanced osteocytic and osteoblastic production of receptor of activated NF- κ B ligand (RANKL) resulting in increased osteoclastic bone resorption that releases Ca $^{2+}$ from the bone (see Klein et al., 2016). One purpose of the increased extracellular Ca $^{2+}$ concentration likely relates to its action as a chemokine on the one hand to recruit macrophages to sites of cell death and on the other to play a role in amplifying the inflammatory response via stimulating the assembly of a cytoplasmic multiprotein complex inflammasome that mediates proinflammatory cytokine maturation by activation of caspase-1 (see Hendy and Canaff, 2016 for further details).

Therefore, the CaSR in parathyroid gland and kidney executes its role in Ca $^{2+}$ homeostasis but it is also expressed in monocytes and macrophages. This allows the CaSR to play critical roles in promoting and mediating the inflammatory response to tissue injury as well as minimizing or limiting these effects via its role in systemic Ca $^{2+}$ homeostasis. Whether there is a place for the use of CaSR allosteric modulators in relevant clinical areas, for example, in reducing the degree of hypocalcemia in critically ill patients, remains to be explored. While extracellular Ca $^{2+}$ is an activator of the NOD-like receptor family, pyrin domain-containing protein-3 (NLRP3) inflammasome, influenza virus infection also activates this particular inflammasome with resultant production of IL-1 β (Allen et al., 2009; Owen and Gale, 2009; Thomas et al., 2009). This may be relevant to the observation that ADH1 patients with gain-of-function CASR mutations often become symptomatic (e.g., with seizures) during periods of intercurrent illness (Hendy et al., 2009). Enhanced expression of the activated CaSR by the increased circulating IL-1 β cytokine would result in extracellular Ca $^{2+}$ levels to drop further to symptomatic levels.

An additional pathological role for Ca $^{2+}$, CaSR, proinflammatory cytokines, and obesity has been suggested (Villaruel et al., 2014). It is proposed that Ca $^{2+}$ activation of the CaSR in white adipose tissue preadipocytes increases proinflammatory cytokine production, proliferation and differentiation but decreased lipid accumulation and adipose tissue dysfunction (Cifuentes et al., 2012). The obesity associated cytokines like TNF- α and IL-1 β increase adipocyte CaSR expression (Cifuentes et al., 2010) by releasing NF κ B to translocate to the nucleus and activate the CASR gene at κ B response elements in its promoters (Canaff and Hendy, 2005). This represents a positive feed forward loop.

Glial Cells Missing-2

In *Drosophila* the transcription factor glial cells missing (*GCM*) acts as a developmental binary switch between glia and neurones. In mammals, there are two orthologs *GCM1/GCMA* and *GCM2/GCMB* critical for parathyroid and placental development, respectively (Kim et al., 1998; Kammerer et al., 1999; Kanemura et al., 1999). The five exons of the *GCM2* gene (OMIM# 603716) on chromosome 6p24.2 encode a protein of 506 amino acids. *GCM2* is expressed in the PTH-secreting cells of the parathyroid glands and is critical for their development

in terrestrial vertebrates, and continues to be expressed in the adult (Günther et al., 2000; Maret et al., 2004; Okabe and Graham, 2004; Liu et al., 2007). From NH₂ to COOH termini of the GCM2 protein there is a DNA-binding domain, transactivation domain 1, an inhibitory domain, and transactivation domain 2.

Homozygous or heterozygous inactivating mutations occur in familial isolated hypoparathyroidism (FIH) inherited in an autosomal recessive or dominant manner, respectively (Hendy and Cole, 2015). Autosomal recessive mutations include missense, stop, frameshift, and gene deletion, (e.g., Ding et al., 2001; Baumber et al., 2005; Thomée et al., 2005; Bowl et al., 2010; Tomar et al., 2010; Doyle et al., 2012). Autosomal dominant mutations include missense and single-nucleotide deletion (e.g., Mannstadt et al., 2008; Canaff et al., 2009b; Mirczuk et al., 2010; Yi et al., 2012). *In vitro* functional studies of some of these mutants have demonstrated loss of GCM response element binding and/transcriptional activity in the case of recessive mutations, as well as the ability of dominant mutants to inhibit activity of wild-type GCM2 when the two are transfected together into cells (Mannstadt et al., 2008; Canaff et al., 2009b; Mirczuk et al., 2010).

Genetic defects affecting GCM2 are rare in FIH: a study of 20 unrelated FIH cases (10 familial and 10 sporadic) found several polymorphic variants, but did not find GCM2 mutations that segregated with the disease and/or led to loss of function (Maret et al., 2008).

The CaSR is an early parathyroid differentiation marker (Liu et al., 2007). Absence or reduction of parathyroid GCM2, as in mice null for *Gcm2* (Günther et al., 2000) or in cultured human parathyroid cells treated with GCM2 siRNA (Mizobuchi et al., 2009), correlates with lack or decreased expression of the CaSR. GCM2 transactivates the *CASR* gene via GCM response elements in promoters P1 and P2 (Canaff et al., 2008a, 2009b). Thus, GCM2 and CaSR are mechanistically linked with respect to the development of the evolutionarily related parathyroid glands (in terrestrial vertebrates) and gills (in fish) (Okabe and Graham, 2004).

v-maf musculo-aponeurotic fibrosarcoma oncogene homolog B (MafB), a transcriptional activator, is present in developing and mature parathyroid glands (Kamitani-Kawamoto et al., 2011). MafB acts downstream of GCM2 and ensures that the developing parathyroid glands properly localize between the thyroid and the thymus (Kamitani-Kawamoto et al., 2011). GCM2 associates with MafB to synergistically activate *PTH* gene expression (Kawahara et al., 2010; Kamitani-Kawamoto et al., 2011).

Haploinsufficiency of the dual zinc-finger transcription factor, GATA3, results in the congenital hypoparathyroidism-deafness-renal dysplasia (HDR) syndrome (Ali et al., 2007). *Gata3* knockout mouse embryos lack *Gcm2* expression and have gross defects in the third and fourth pharyngeal pouches including absent parathyroid-thymus primordia (Grigorieva et al., 2010). GATA3 transactivates the *GCM2* gene by binding specifically to a double-GATA-motif within the *GCM2* promoter. In addition, GATA3 cooperates with GCM2 and MafB to activate *PTH* gene expression by interacting with the ubiquitous specificity protein-1 (SP1) transcription factor (Han et al., 2015). Thus, GATA3, GCM2 and MafB are part of a critical transcriptional cascade in

the parathyroid morphogenesis and CaSR and PTH expression pathway (Grigorieva and Thakker, 2011; Han et al., 2015).

The persistence of GCM2 expression in the adult parathyroid raises the question of whether its overactivity or reduced expression could play a role in the development of parathyroid hyperfunction or tumorigenesis. In support of the first notion, increased expression of GCM2 has been noted in some adenomas of primary parathyroid patients (Kebebew et al., 2004). Furthermore, the allele frequency of a common Y282D polymorphism is significantly higher in Italian cohorts of primary hyperparathyroid patients than in normal individuals and the 282D variant exhibits higher activity on a GCM element promoter than Y282 (D'Agruma et al., 2014). In support of the second supposition, reduced levels of GCM2 mRNA have been reported in adenomatous tissue from some patients with primary hyperparathyroidism (Correa et al., 2002). This may contribute to the reduced CaSR expression commonly found in these tumors that might itself contribute to dysregulated control of parathyroid cell proliferation.

CASR Expression and Kidney Stones

Evidence has been provided that CaSR polymorphisms such as R990G can increase the susceptibility to hypercalciuria and urolithiasis (Liu et al., 2015). But single nucleotide polymorphisms (SNPs) within the regulatory regions of the *CASR* gene have also been associated with kidney stone risk, for example, in primary hyperparathyroidism (Vezzoli et al., 2011). A comparison of idiopathic calcium stone formers and healthy controls revealed one SNP (rs6776158 [A>G]) located in the *CASR* P1 promoter that was associated with nephrolithiasis (Vezzoli et al., 2013). Sr²⁺ handling has been proposed to be a mirror of Ca²⁺ metabolism and reduced Sr²⁺ excretion after an oral load was observed in GG homozygous stone formers. Activity of the G allele *CASR* P1 promoter-luciferase reporter was lower (than the A allele promoter) in cell transfection studies and CaSR mRNA levels were lower in kidney medulla samples in GG homozygous individuals than A allele carriers (Vezzoli et al., 2013). Therefore, the minor allele at rs6776158 may predispose to calcium stones by decreasing transcriptional activity of the *CASR* P1 promoter and CaSR expression in kidney tubules. Claudin-14 is a member of a superfamily of proteins that regulate paracellular transport of ions and small solutes at epithelial tight junctions. A mechanistic link between *CASR* gene activity was suggested by the finding that claudin-14 mRNA levels were lower in the *CASR* rs6776158 GG homozygous subjects. Therefore, in these cases the predisposition to calcium nephrolithiasis is by a mechanism independent of hypercalciuria (Vezzoli et al., 2013). This hypothesis received further support by a comparison of another *CASR* gene regulatory SNP and R990G with respect to stone risk in primary hyperparathyroidism (Vezzoli et al., 2015).

In a genome-wide association study (GWAS) of Icelandic kidney stone cases a suggestive association of (rs7627468 [A]) in a *CASR* intron1 regulatory region was found in a large population set (Oddsson et al., 2015). A strong association was found for a polymorphism (rs199565725 [delAC]) in intron 1 of the *CLDN14* gene encoding claudin-14 that might mediate a decrease in *CLDN14* gene function. These data reinforced that of the same

group in an earlier study (Thorleifsson et al., 2009). The authors observed uncorrelated genome-wide significant association of variants at the *CASR* locus that also influence biochemical traits, serum total and ionized calcium, for example, but do not associate with the kidney stone disease. This implies that the risk is not mediated solely through the serum level of calcium or other biochemical trait (Oddsson et al., 2015), and provides further indirect support for the *CASR* directed Claudin-14 mechanism (Gong et al., 2012; Gong and Hou, 2014; Toka et al., 2015; Riccardi and Valenti, 2016).

CASR and Epigenetic Modification

Altered CaSR expression occurs in benign and malignant tumors suggesting it as either a tumor suppressor or oncogene. The epigenetic inactivation of the *CASR* may underlie the reduced CaSR expression in some cancers. The *CASR* P2 promoter that is GC-rich, is methylated to a greater extent in colorectal tumors relative to adjacent mucosa and this correlates with the reduced CaSR levels in the tumors (Hizaki et al., 2011; Fetahu et al., 2014a). Use of histone deacetylase inhibitors in colon cancer cell lines most of which poorly express CaSR has implicated the involvement of H3K9 deacetylation in the silencing of *CASR* in colorectal cancer (Hizaki et al., 2011; Fetahu et al., 2014a). Similar findings with respect to methylation of the *CASR* P2 promoter were reported for unfavorable neuroblastomas in which CaSR is barely detectable (Casalà et al., 2013). In addition, monosomy of chromosome 3 where *CASR* resides was found in the vast majority of primary neuroblastic tumors of all types (Casalà et al., 2013). Hence the *CASR* is silenced by both genetic and epigenetic means in these tumors. However, no evidence has been found of loss of *CASR* alleles (Farnebo et al., 1997) or *CASR* gene methylation being responsible for the reduced CaSR levels in primary and secondary hyperparathyroidism (Hofman-Bang et al., 2012; Sulaiman et al., 2013; Varshney et al., 2013). Moreover, the molecular mechanisms underlying the enhanced expression of CaSR in breast and prostate tumors that implicates an oncogenic role for CaSR in these tissues have yet to be deciphered (Tennakoon et al., 2016).

CaSR And MicroRNAs

MicroRNAs (miRNAs) are short non-coding RNAs that function in RNA silencing and post-transcriptional regulation of gene expression. A further cause of silencing of the CaSR in colorectal tumors has been proposed to be increased expression of miR-135b and miR-146b that are considered to be oncogenic (Fetahu et al., 2016). In colon cancer cell lines other miRNAs, miR-21, miR-145, and miR-135a, are inversely correlated with CaSR expression (Singh and Chakrabarty, 2013; Singh et al., 2013). Altered expression of miRNAs may well have an important role

in development of parathyroid tumors and miRNAs have been implicated in parathyroid function. Dicer-dependent maturation of parathyroid miRNAs is responsible for the dysregulated control of PTH secretion in a uremic mouse model of secondary hyperparathyroidism (Shilo et al., 2015). However, specific miRNAs that directly affect parathyroid CaSR expression have yet to be identified.

PERSPECTIVES AND CONCLUSIONS

The CaSR is expressed in the central nervous system and the roles it may play in the brain are being explored (Ruat and Traiffort, 2013). It will be important in future to understand the precise mechanisms underlying the postnatal upregulation of the brain CaSR during development and whether altered regulation of the CaSR plays a role in some cases of epilepsy. Within neurons the CaSR may play a role in susceptibility to Alzheimer's Disease (AD) and its progression (Chiarini et al., 2016). *CASR* expression may be altered in AD as it is regulated by the same transcription factors (e.g., SP1/3, AP1, STAT1/3, NF- κ B, TFIID) already known to modulate other AD-regulated genes (Chiarini et al., 2016). However, this has yet to be directly evaluated.

The blood-brain barrier defends extracellular Ca^{2+} in the brain from changes in serum Ca^{2+} and at rest brain extracellular Ca^{2+} is maintained at 1.1 mM. During neuronal activity extracellular Ca^{2+} can fall sharply as Ca^{2+} moves to intracellular compartments. Following action potentials, Ca^{2+} can fall as low as 0.3 mM in the synaptic cleft (Jones and Smith, 2016). Any potential changes on *CASR* gene expression and their consequences have yet to be examined.

Further insights to those described in the present review, can be anticipated with respect to identification of additional transcriptional factors and their cis-elements in the *CASR* gene promoters, epigenetic changes involving direct methylation of the *CASR* DNA as well as histone modifications and chromatin remodeling. The direct effect of miRNAs on degradation of CaSR mRNA or inhibition of its translation will become clearer.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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