The 2nd International Symposium on the Calcium-sensing Receptor

March 3 - 4, 2015

Gallery 2, Meeting Room
Omni Hotel
San Diego, California
Dear Meeting Attendees,

On behalf of the Organizing Committee, we would like to welcome you to The 2nd International Symposium on the Calcium-sensing Receptor at the Omni Hotel in San Diego, California. The program will feature recent research on the role of the CaSR in skeletal development and physiology, vascular and pulmonary functions, neuronal injury, neuroendocrine control mechanisms, parathyroid physiology, mineral and energy metabolism, exocrine and transport functions in the gut and kidney, and cancer cell signaling. Clinical uses of calcimimetics and calcilytics and translational research exploring potential new therapeutic uses of CaSR modulators are highlights.

In addition to senior experts presenting their work, we have a number of Young Investigators that will be presenting their research. This promises to be an exciting forum for scientists from different disciplines across the globe to collaborate, learn, and present information on the calcium-sensing receptor and its role in the body.

Sincerely,

Co-chairs of Organizing Committee,

Wenhan Chang, PhD
Research Scientist
San Francisco Veterans Affairs Medical Center
Professor of Medicine
University of California, San Francisco

Dolores Shoback, MD
Professor of Medicine,
University of California, San Francisco
Endocrine Research Unit – 111N
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Edward F Nemeth, PhD
MetisMedica
Speaker Abstracts
Physiological Implications of Calcium Sensing Receptor Trafficking as a Regulator of Signaling

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Calcium sensing receptors (CaSR) translate changes in extracellular calcium into short and long term cellular responses. CaSR signaling is regulated by a feed forward mechanism which relies on signaling-activated trafficking through the secretory pathway to control plasma membrane levels of CaSR. Net CaSR signaling shows little functional desensitization. Both trafficking and enhanced receptor synthesis are maintained during prolonged elevations in extracellular calcium. The feed-forward mechanism which maintains CaSR signaling under physiological conditions may contribute to pathology in the chronic presence of elevated extracellular calcium. We therefore explored possible mechanisms which down-regulate CaSR signaling by attenuating trafficking. We considered the model provided by keratinocyte differentiation, in which elevated extracellular calcium leads to up-regulated expression of an exon 5-deleted form of CaSR, CaSREdx5. CaSREdx5 forms heterodimers with full length CaSR and acts as a dominant negative to prevent exit of receptors from the endoplasmic reticulum. Coexpression of full length and CaSREdx5 blocks maturation of full length CaSR, attenuating CaSR signaling. Retention mechanisms for CaSREdx5 are unique, and do not depend on carboxyl terminal binding of 14-3-3 proteins. Culture of confluent aortic endothelial or vascular smooth muscle cells in elevated calcium or [calcium x phosphate] for extended periods induces calcification. During development of calcification, we observe up-regulation of expression of CaSREdx5 at both message and protein levels. Staining with an antibody selective for the exon 5-deleted form of CaSR revealed significant up-regulation of CaSREdx5 in human femoral arteries with calcium. The major implication of these studies is that up-regulation of CaSREdx5
may be an integral regulatory response to attenuate chronic CaSR signaling under pathological states resulting from elevated extracellular calcium. Supported by Geisinger Clinic.
MOLECULAR BASIS OF RECEPTOR-MEDIATED EXTRACELLULAR CALCIUM SIGNALING

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We have reported several potential calcium-binding sites located within the calcium sensing receptor (CaSR)’s extracellular domain (ECD) using our developed computational algorithms. Here, we report the central role of predicted Ca2+-binding Site 1 within the hinge region of the ECD of CaSR and its interaction with other Ca2+-binding sites within the ECD in tuning functional positive homotropic cooperativity caused by changes in [Ca2+]o. An adjacent L-Phe-binding pocket that is responsible for positive heterotrophic cooperativity between [Ca2+]o and L-Phe in eliciting CaSR-mediated [Ca2+]i oscillations is also identified. The hetero-communication between Ca2+ and L-Phe globally enhances functional positive homotropic cooperative activation of CaSR in response to [Ca2+]o signaling. We next purified the glycosylated ECD, and demonstrated its direct interaction with Ca2+ and Phe as well as the resultant impact on its structure and associated conformational changes. By comparing human prostate cancer tissue sections in microarrays, we found that the CaSR was expressed in both normal prostate and primary prostate cancer as assessed by immunohistochemistry. Metastatic prostate cancer tissue obtained from bone had higher CaSR expression than primary prostate cancer within the prostate gland. The point of emphasis of the present study is that our findings provide new insights into the mechanisms by which Ca2+ and amino acids regulate the CaSR and the receptor may have a role in promoting bony metastasis of prostate cancer, hence raising the possibility of reducing the risk of such metastases with CaSR-based therapeutics.
EVOLUTIONARY HISTORY OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR (CaSR)

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The extracellular calcium-sensing receptor (CaSR) is expressed and is functionally active across the major jawed vertebrate groups, and genomic data suggests the existence of CaSR-like proteins in the jawless vertebrates and non-vertebrate chordates. The highly-conserved CaSR family of proteins is related phylogenetically to pheromone, odorant, taste and vomeronasal receptors of the glutamate receptor-like family of the GPCR superfamily. Despite the strong evolutionary conservation among vertebrate CaSRs, especially in structural domains and motifs that are important for functional activity, taxon-specific differences in both primary and higher-order structures distinguish CaSRs of cartilaginous fishes, bony fishes and tetrapods. Evidence of evolutionary selection along the branches leading to major vertebrate clades suggests that the distinctive differences in CaSR proteins among taxonomic groups may be related to differences in the physiological mechanisms for calcium homeostasis, and to characteristic life history features of the various groups. Physiological and life history differences are seen in patterns of bone biology and skeletogenesis, and in the form and availability of environmental calcium as it relates to hydromineral homeostasis. In mammals, CaSR structures and biological roles have been well studied in health and disease, but in fishes, until recently, little has been known. Based on phylogenomic and phylogenetic analyses, comparative protein homology modeling and functional expression of non-mammalian CaSRs, the evolutionary history of CaSRs and the functional significance of structural differences can be revealed. The major point of emphasis of the present study is that vertebrate CaSRs have a deep origin in the chordate-vertebrate lineage, and that they have experienced overall strong purifying selection with bouts of positive (adaptive) evolutionary selection along the branches to major clades.
CALCIUM-SENSING RECEPTOR: 
REGULATION OF GENE EXPRESSION

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The human calcium-sensing receptor (CaSR) is encoded by a single copy gene – spanning ~103-kb and having 8 exons – on chromosome 3q. Promoters P1 and P2 drive transcription of exons 1A and 1B, respectively, encoding alternative 5′-UTRs that splice to exon 2 encoding the common part of the 5′-UTR. Exons 2-7 encode the CaSR protein of 1078 amino acids. Promoter P1 has TATA and CCAAT boxes upstream of the transcription start site, whereas promoter P2 has Sp1/3 motifs at its start site. The exon 1A transcripts are reduced in parathyroid tumors and colon carcinomas consistent with their reduced CaSR protein expression. Functional elements responsive to 1,25-dihydroxyvitamin D (1,25D), proinflammatory cytokines, and the transcription factor glial cells missing-2 (GCM2) are present in the CASR promoters. Vitamin D: 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. Treatment of autosomal dominant hypocalcemia (ADH) type 1 patients with vitamin D may further stimulate the overactive CaSR leading to nephrocalcinosis and nephrolithiasis. Proinflammatory cytokines: The hypocalcemia, dysregulated parathyroid hormone (PTH) levels, reduced 1,25D levels and increased calcitonin precursors in critically ill patients with sepsis or burn injury may be related to upregulation of CASR gene by proinflammatory cytokines, TNF-alpha, IL-1beta, and IL-6, via NF-kappaB, and Stat1/3 and Sp1/3, elements in the CASR gene promoters, respectively. The same mechanism may also provoke the symptomatic hypocalcemia and seizures that bring ADH patients with activating CaSR mutations to clinical attention during periods of intercurrent illness. GCM2: The CaSR is transactivated by GCM2 – the transcription factor essential for development of the PTH-secreting cells in the parathyroid gland. Continued expression of GCM2 in the adult parathyroid raises the possibility of its overactivity or reduced expression being associated with parathyroid hyperactivity or tumorigenesis. Summary: The “calciostat” is altered at the gene level under physiological and pathophysiological conditions.
BIASED AGONISTS OF THE CALCIUM-SENSING RECEPTOR

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The calcium-sensing receptor (CaSR) is a G protein-coupled receptor activated by cations like Ca2+, Ba2+ and Sr2+, polyamines like spermine and spermidine, and aminoglycosides like neomycin and tobramycin. The CaSR is a promiscuous G protein-coupled receptor being able to activate several different signaling pathways. CaSR directly couples to the G proteins Gq/11, Gi/o and G12/13 and in rare cases Gs. In addition, the receptor couples to activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) via the Gq/11 and Gi/o proteins, and via β-arrestin recruitment. A new concept in G protein-coupled receptor is biased signaling in which different ligands selectively affect different signaling pathways, presumably by selectively stabilizing different conformations of the receptor. Given the broad range of ligands and signaling pathways engaged by the calcium-sensing receptor we and others have used this receptor as a model system to investigate biased signaling. A prerequisite for performing such studies is to be able to study multiple signaling pathways in an effective fashion. To this end we have studied a HEK-293 cell line stably expressing the rat CaSR and rat medullary thyroid carcinoma 6-23 cells endogenously expressing the CaSR using HTRF assays of the Gq/11, Gi/o and ERK1/2 pathways (Cisbio). The major finding of these studies are that we identified Ba2+, Sr2+, spermine, neomycin, and tobramycin act as biased agonist in terms of efficacy and/or potency.
BIASED SIGNALLING FROM THE CALCIUM-SENSING RECEPTOR (CaSR)

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‘Ligand biased signalling’ (also referred to as biased agonism or functional selectivity) is the phenomenon by which distinct ligands stabilise preferred receptor conformations, with each subset of conformations having the capacity to signal via distinct intracellular pathways. The CaSR responds to multiple endogenous agonists and allosteric modulators and is therefore subject to biased signalling, depending on the agonist and/or modulator that it binds. Interestingly, the CaSR’s ‘natural’ bias is altered in pathophysiological states, such as those arising from the many naturally occurring CaSR mutations that have been identified. Although these mutations have traditionally been classified as “loss-” or “gain-of-function” mutations, they do not necessarily cause a universal loss or gain in receptor coupling to all its intracellular signalling partners. Thus, they can alter the receptor’s natural signalling preference. Furthermore, small molecule calcimimetics and calcilytics engender biased allosteric modulation of the CaSR's downstream signalling pathways. They can also differentially modulate signalling versus trafficking of the CaSR, which may be taken advantage of when treating patients with loss-of-expression CaSR mutations. Thus, biased signalling from the CaSR has important implications both pathophysiologically and therapeutically.
CALCIUM-SENSING RECEPTOR AND THE PARATHYROID: PAST, PRESENT AND FUTURE

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The cloning of the calcium-sensing receptor (CaSR) and the demonstration that mutations of the CaSR underlie well-recognised inherited disturbances of calcium metabolism by Brown, Hebert, and colleagues solved a key problem in parathyroid physiology: the identity of the receptor protein that mediates extracellular calcium (Ca2+o) sensing. Thus, the CaSR mediates negative feedback regulation of parathyroid hormone (PTH) secretion by extracellular calcium (Ca2+o). It also mediates Ca2+o-dependent control of parathyroid cell proliferation and differentiation, and is likely to mediate Ca2+o-dependent control of PreProPTH gene expression. Furthermore, as first demonstrated by Nemeth and colleagues, CaSR-acting pharmacological modulators known as the calcimimetics and calcilytics may be used to respectively suppress or elevate serum PTH levels with application to the treatment of disorders of calcium and/or bone metabolism. Although CaSR-dependent signaling has been studied in detail in cell lines that have been transfected with the CaSR, as well as parathyroid cell models, the signaling mechanisms that underlie the control of key downstream events such as PTH secretion are not well understood and represent a necessary focus of future research. Our own research focuses on the role of the CaSR in the control of the local synthesis of a key modulator of parathyroid cell function, 1,25-dihydroxyvitamin D, as well as the nature of the intrinsic mechanisms that support PTH secretion and are subject to negative modulation by the CaSR.
CASR AND PARATHYROID CELL SIGNALING

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CaSR signaling involves the heterotrimeric G proteins, Gq/11, Gi/o and G12/13 and direct interactions with signaling scaffold proteins. With such an array of potential effector pathways, whose components will differ stoichiometrically between cell-types, CaSR signaling may depend both on its cell-type/subcellular location (caveolae in parathyroid cells) and on its potential bias for particular ligands. Mice with a PT-specific lack of Gaq/11 develop neonatal severe primary hyperparathyroidism, while Ga11 loss- or gain-of-functions mutations in humans can increase or decrease parathyroid hormone (PTH) secretion respectively. Such CaSR-induced, IP3-mediated Ca2+i mobilization may drive Ca2+-dependent phosphodiesterase activity to lower cAMP levels and suppress PTH secretion. CaSR activity is regulated both extracellularly, e.g. by pathophysiologic pHo changes, and, intracellularly by phosphorylation. Protein kinase C-mediated CaSRT888 phosphorylation inhibits Ca2+o-induced Ca2+i mobilization, overcoming the inhibitory effect of high Ca2+o thus permitting increased PTH secretion. This is mimicked by the phosphatase inhibitor calyculin-A which prevents CaSRT888 dephosphorylation. The clinical mutation CaSRT888M is not susceptible to such inhibitory feedback and is thus gain-of-function, resulting in tonic PTH suppression (autosomal dominant hypocalcemia). Finally, CaSR-mediated parathyroid transcriptional control remains poorly understood but may involve CaSR’s stimulatory effects on the MAP kinases (ERK, p38 and JNK), Rho kinase, Wnt-b-catenin and CREB. Also, CaSR couples several C-terminal binding partners and signaling scaffolds, most notably filamin and the clathrin-coated vesicle component, adaptor protein-2.
In vitro cell cultures of parathyroid cells have long represented an issue of key importance for several researchers who have aimed their studies at the physiology and pathologies of this endocrine gland. In vitro parathyroid cell culture has proved to be challenging, presenting several difficulties to be overcome. Bovine parathyroid glands and human parathyroid glands have been extensively used to develop in vitro cell models of parathyroid cells. Human pathologic parathyroid glands, such as parathyroid adenomas and hyperplastic parathyroid glands from patients with secondary hyperparathyroidism due to chronic kidney disease constitute the models most commonly used for the development of parathyroid cell systems. On the other hand, normal human parathyroid glands have been used less commonly to establish parathyroid cell cultures, because they have proved to be more difficult to grow in culture due to their very low proliferative activity. Similarly, reports of parathyroid cell cultures derived from parathyroid carcinomas are scarce in literature given the very low frequency of parathyroid cancer. Most of the in vitro parathyroid cell models described in the literature are primary cultures, which are only viable for a short period of time, while there are only a few reports regarding the achievement of long-term viable parathyroid cell cultures or cell lines. The lack of a continuous cell line of epithelial parathyroid cells able to produce PTH has hampered the studies on in vitro evaluation of the mechanisms involved in the control of parathyroid cell function and proliferation. The PT-r cell line was first established from rat parathyroid tissue in 1987, but these cells were known to express the parathyroid hormone-related peptide gene, but not the Pth gene. In an attempt to subclone the PT-r cell line, a rat parathyroid cell strain was isolated and named PTH-C1. PTH-C1 cell line produces PTH and expresses the calcium sensing receptor (Casr) gene and other genes known to be involved in parathyroid function. Most importantly, the PTH-C1 cells also exhibit an in vitro secretory response to calcium. Altogether these findings indicate the uniqueness of the PTH-C1 cell line as an in vitro model for cellular and molecular studies on parathyroid physiopathology.
PARATHYROID GABABR1 IS THE MOLECULAR BASIS FOR THE DEVELOPMENT OF HYPERPARATHYROIDISM DUE TO CaSR DEFICIENCY

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Mechanisms causing PTH hypersecretion in conditions of primary hyperparathyroidism (HPT) due to CaSR inactivity remain largely unclear. We found that the CaSR and the type B gama-aminobutyric acid (GABA) receptor 1 (GABABR1), another member of family C GPCR, physically interact to suppress the expression and signaling ability of their counterparts in cDNA-transfected HEK-293 cells. Parathyroid cells (PTCs) express protein and RNA of the GABABR1 and the 65 kD glutamic acid decarboxylase (GAD65), a critical enzyme for GABA synthesis. Adding GABA or baclofen, a GABABR1 agonist, to intact parathyroid glands (PTGs) cultured from wild-type mice increases their maximal PTH secretion (PTH-Max) and shifts their Ca2+-setpoints to the right, indicating enhanced secretory capacity and reduced Ca2+-responsiveness of the glands. To assess the role of GABABR1 in mediating PTH secretion in vivo at basal and HPT states, we studied PTCCaSR1-Hom mice with both of their Gabbr1 genes ablated specifically in PTCs and mice with Gabbr1 and Casr double gene KO (DKO). Heterozygous (PTCCA-SR-Het) and homozygous (PTCCA-SR-Hom) PTC-specific CaSR KO elevated serum PTH (sPTH) and calcium (sCa) levels, recapitulating phenotypes of human Familial Hypocalciuric Hypercalcemia (FHH) and neonatal severe HPT (NSHPT), respectively. The PTCCA-SR-Het mice are viable, but PTCCA-SR-Hom mice die before 3-4 weeks of age. In contrast, the PTCGABABR1-Hom mice show reduced sPTH and sCa levels. Furthermore, concurrent GABABR1 KO rescues the early lethality of PTCCA-SR-Hom mice and suppresses the sPTH and sCa levels in the PTCCA-SR-Hom //GABABR1-Hom DKO mice. Similarly, GABABR KO suppresses the sPTH and sCa levels in the PTCCA-SR-Het//GABABR1-Hom DKO mice. PTGs cultured from the PTC-CaSR-Het mice showed a profound increase in PTH-Max by 3-4 fold and a right-shift in Ca2+-setpoint from 1.3 to 1.5 mM vs controls, but these effects were completely abrogated by concurrent
ablation of GABABR1 in the PTGs of the PTCCaSR-Het//GABABR1-Hom DKO mice. Our data support the concept that parathyroid GABABR1 critically controls mineral homeostasis at basal and HPT states by sustaining tonic PTH secretion and by altering Ca2+-responsiveness of PTCs, providing a molecular basis for the development of HPT.
EXTRAPARATHYROID CaSR CONTROLS BLOOD CALCIUM CONCENTRATION INDEPENDENTLY OF PARATHYROID HORMONE SECRETION

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The extracellular Ca2+ -sensing receptor (CaSR) expressed by parathyroid cells controls blood calcium concentration by regulating parathyroid hormone (PTH) secretion. However, CaSR is also expressed in other organs, including the kidney. We investigated the role of extraparathyroid CaSR selectively increased renal tubular calcium absorption and blood calcium concentration independent of change in PTH secretion and without altering intestinal calcium absorption. CaSR inhibition increased blood calcium concentration in animals pretreated by a biophosphonate, indicating that the increase did not result from release of bone calcium. As measured by in vitro microperfusion of cortical TAL, CaSR inhibitors NaCl reabsorption. Both in vivo and ex vivo, opposite results were obtained using cinacalcet or NPS-R568, two activators of CaSR. We conclude that CaSR is a direct determinant of blood calcium concentration, independent of PTH, which modulates renal tubular calcium transport in the TAL via the permeability of the paracellular pathway. These findings suggest that CaSR inhibitors may provide a new specific treatment for disorders related to impaired PTH secretion, such as primary hypoparathyroidism.
LESSON LEARNED FROM THE KIDNEY-SPECIFIC CASR ABLATION

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Calcium-sensing receptor (Casr) gene mutations can lead to abnormal urinary calcium (Ca) excretion in the context of parathyroid hormone (PTH)-dependent variations in serum Ca level. The role of Casr in the kidney is less well understood. We therefore ablated renal Casr utilizing mice expressing Cre recombinase under the Six2 promoter. Renal tubule Casr-deficient mice (rtCasr/-) showed no detectable levels of Casr mRNA and protein in the kidney. Baseline serum Ca, phosphorus, magnesium (Mg), and PTH levels were unchanged. However, rtCasr/- mice featured significantly lower urinary Ca excretion under dietary Ca supplementation (urinary Ca-to-creatine, 0.31±0.03 vs 0.63±0.14; $P=0.001$). Claudin14, a negative regulator of paracellular cation permeability in the thick ascending limb (TAL), was significantly downregulated by ~80% on mRNA level, whereas Claudin16 was slightly but significantly upregulated. Immunoblotting on whole-kidney lysates suggested increased activation of the Na+K+2Cl- cotransporter NKCC2. However, preliminary data measuring transepithelial transport of Na+ and Cl- across isolated cortical TAL tubules, do not support increased NaCl transport in rtCasr/- mice. The point of emphasis of the present study is that the role of Casr in the kidney is the inhibition of Ca reabsorption. Renal Casr regulates Ca reabsorption in the TAL, independent of any change in PTH, by modulating paracellular Ca transport through altering the expression of claudin 14 and 16.
THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR (CaSR) ACTIVATES adam10 TO CLEAVE MEMBRANE-BOUND KLOTHO

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Klotho, the anti-aging protein exists primarily as membrane-bound and circulating or soluble forms. Soluble Klotho (sKl) is responsible for Klotho's systemic anti-aging effects, and the primary source of sKl is the kidney. The mechanism by which sKl protects against the premature aging phenotype is not defined, nor are the mechanisms by which it is regulated physiologically. Serum levels are derived from cleavage of the membrane-bound form by proteases such as ADAM10 and ADAM17.

Klotho is expressed in the proximal tubule where with the FGFR1 receptor, it binds FGF23 to regulate PO4 transport and vitamin D metabolism. The highest levels of renal Klotho expression are on the BLM of the DCT, but its role in this segment is not well defined. We found that the CaSR, a GPCR that is highly expressed in the DCT interacts with ADAM-10 in the yeast two hybrid system, heterologous expression systems, and in kidney extracts, suggesting that the CaSR could regulate cleavage to Klotho by ADAM10. Using expression of full length Klotho, the CaSR, and ADAM10 in HEK-293 and IMCD-3 cells, we found that medium Klotho increased in response to CaSR activation by Ca, the calcimimetic R568, and ADAM10 expression, and that this increase is inhibited by TAPI-1, an ADAM inhibitor. Additionally, the CaSR co-immunoprecipitates with the full length Klotho, but not the shorter secreted form, as well as ADAM10. The CaSR coimmunoprecipitates with ADAM10 and Klotho from kidney extracts. Finally, the calcimimetic R568 and increased Ca levels increased medium Klotho levels from kidney slices and homogenates, while Ca-mediated increases were blocked by the calcialyitc NPS2143. These results indicate that the CaSR in the distal nephron may be responsible for physiologic regulation of serum Klotho levels via ADAM10.
THE CALCIUM-SENSING RECEPTOR IN THE INTESTINE. EVIDENCE FOR REGULATION OF COLONIC ABSORPTION, SECRETION, PERMEABILITY AND IMMUNITY

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The CaSR is expressed by several types of cells in the gut, suggesting it may play multiple roles in regulating intestinal functions. Previous studies have shown that activation of CaSR in colonic epithelial cells by calcium, spermine or R568, abrogates fluid secretion induced by secretagogues. Our recent studies using pH-stat technique showed that activation of this same receptor by R568 suppressed secretagogue-induced HCO3 secretion mediated by CFTR, but it stimulated HCO3 secretion and solute absorption mediated by Cl/HCO3 and SCFA/HCO3 exchanges when secretagogue stimulation was absent; both effects were absent in intestinal epithelium-specific CaSR KO mice. CaSR is also expressed in the enteric nervous system (ENS), a regulator of secretion and motility. By measuring short-circuit current responses to R568 in ENS-containing colonic segments isolated from wild type and neuron-specific CaSR KO mice and by comparing their fluid secretory responses in vivo, we found that neuronal CaSR acted to suppress ENS activity and ENS-mediated electrolyte secretion. Finally, by characterizing gut epithelial-specific CaSR KO mice and colonic inflammatory responses to dietary CaSR agonists, we demonstrated that this CaSR also played a critical role in maintaining epithelial permeability and barrier function, normal microbiota composition, and mucosal immune responses. Compared to wild type mice, the CaSR/- mice were significantly more prone to chemically induced intestinal inflammation resulting in colitis. Together, our results suggest that intestinal CaSR may provide a novel therapeutic target to treat both secretory and inflammatory diarrheal disorders, including infectious diarrhea, inflammatory bowel disease and irritable bowel syndrome.
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THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR (CaSR) AND INTESTINAL PARACRINE INTERACTION

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The CaSR is present both on intestinal epithelia and sub-epithelial myofibroblasts which are physically juxtaposed to signal to the epithelia. Activating the CaSR on myofibroblasts stimulates synthesis and secretion of BMP2, IL-11, HGF, Wnt5a, and the Wnt antagonist, Dkk1. CaSR stimulation of the overlying epithelia generates the receptors for each of these ligands (BMPR1, IL-11Ra, cMet, Ror2 and LRP6). These paracrine effects increase epithelial barrier capacity (BMP2, IL11, Dkk1), accelerate restitution (BMP2, HGF, Wnt5a), stimulate differentiation (Wnt5a) or reduce inflammation (Wnt5a/Ror2) by activating deneedylation of Cullin1. CaSR stimulated Dkk1 also interacts with Krm2/Ror2 to titrate Wnt5a effects. CaSR activation dephosphorylates Wntless (GPR177), which increases Wnt5a secretion but inhibits Wnt3a secretion. Phenotypes of “rescued” CaSR/PTH intestine demonstrate increased canonical Wnt signaling (Wnt3a) and reduced non-canonical Wnt (Wnt5a) signaling. Enteroids of C-/P- crypts grown in Matrigel demonstrate both epithelial and stromal Wnt requirements. Hypertrophy of both the heart and neuroepithelial bodies of the lung are found in the C-/P- mice. The major finding of this work is that CaSR will alter which Wnt family ligand is secreted which generates distinct paracrine effects on tissue homeostasis.
THE ROLE OF CaSR IN BONE HOMEOSTASIS

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CaSR not only modulates calcium homeostasis by its role in the parathyroid and kidney but also appears to regulate skeletal metabolism. Thus, CaSR in chondrocytic cells may be a part of a network including serum phosphorus, PTHrP, and 1,25(OH)2D that co-ordinate development of the cartilaginous growth plate in the growing skeleton. During early development and during transient increases of circulating PTH, CaSR activation in osteoblasts appears necessary for osteoblast proliferation, differentiation and bone matrix production. CaSR activation appears to inhibit osteoclast activity in early development, resulting in uncoupling of bone resorption from formation leading to more effective bone anabolism. In more mature animals CaSR increases osteoblast activity and bone formation but also augments osteoblastic RANKL and osteoclastic bone resorption, resulting in increased bone turnover that can amplify the effects of elevated circulating PTH to increase bone resorption. The interaction with PTH may lead, respectively, to net bone formation in trabecular bone or resorption in cortical bone. Activation of osteoblast CaSR enhances bone matrix mineralization likely in association with other regulators on mineralization. By altering PTH levels and improving calcium homeostasis, allosteric modulators of the CaSR are useful in some clinical conditions, however further understanding of the molecular mechanism of CaSR action in bone cells may provide improved CaSR based drugs which may prove useful in metabolic bone diseases.
CaSR AND OSTEOCLASTS FUNCTIONS

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Throughout life a continuous process of bone turnover takes place, involving the degradation of old bone and formation of new bone. The bone remodeling process is the result of a collaborative effort of osteoclasts, cells responsible for bone resorption, and osteoblasts, cells in charge of bone formation. The first stage of the remodeling process is believed to involve the recruitment of monocytic precursors from the blood flow. These cells then infiltrate the bone lining cell layer, followed by the fusion of osteoclast precursors, thus forming multinucleated osteoclasts, capable of bone resorption before they die by apoptosis. Since the concentrations of calcium can reach very high levels near resorbing bone (up to 40 mM), it has been speculated fifteen years ago that calcium and the calcium sensing receptor (CaSR) were involved in one of the signals which initiates and controls the bone renewing process. Indeed, the CaSR has been found to be expressed in osteoclast precursor cells, pre-osteoclasts and osteoclasts in vitro and in vivo. While CaSR independent effects should not be underestimated, migration and differentiation of osteoclast precursor cells as well as osteoclast bone resorption and apoptosis has been shown to be regulated throughout the CaSR activation, leading to a reduced bone resorption in a whole. This brings to the concept that the CaSR expressed in bone cells plays a functional role in bone physiology and pathology, and therefore represents a therapeutic target in diseases or conditions that can cause bone loss.
Calcium-Sensing Receptor-Mediated Regulation of Cell Proliferation and Interactions with the Bone Marrow Microenvironment in Multiple Myeloma

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The calcium sensing receptor (CaSR) plays a crucial role in the localization of hematopoietic stem cells (HSCs) within the endosteal region of the bone marrow (BM), where the stem cell niche is located. However, the functional role of the CaSR in pathologic hematopoiesis is poorly understood. One hematological malignancy in which the CaSR potentially plays an important role is multiple myeloma (MM). MM is a B-cell malignancy characterized by the outgrowth of plasma cells in the BM, with accompanying bone destruction involving bone resorption due to excessively active osteoclasts. This leads to heightened levels of extracellular calcium. To investigate the functional roles of the CaSR in MM, we used genetic loss of function and gain of function studies. Downregulation of the CaSR on MM cells resulted in an impairment of cell proliferation, specific cell migration towards stromal cell-derived factor-1α (SDF-1α), and cell adhesion to fibronectin in vitro. In contrast, overexpression of CaSR resulted in enhanced cell proliferation associated with increased cell cycle entry into the S phase. Although specific cell migration towards SDF-1α was enhanced with CaSR overexpression, there was a decrease in cell adhesion to fibronectin, suggesting that the CaSR is partially involved in mediating adhesive interactions of MM cells and the BM extracellular matrix (ECM). The CaSR also plays a chemoprotective role in MM cells, as CaSR knockdown sensitizes MM cells to bortezomib treatment while CaSR overexpression protects MM cells from bortezomib treatment. Collectively, these data suggest that the CaSR play a crucial role in mediating interactions between MM cells and the BM microenvironment and can potentially be targeted for chemotherapeutic treatments in MM.
THE UPSTREAM AND DOWNSTREAM OF CaSR-DEPENDENT AKT SIGNALING IN HUMAN OSTEOBLASTS

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Akt is a major target of all activated GPCRs in mammalian cells. Our work has focused on understanding the activation of Akt via CaSR in human osteoblasts. The downstream effects of Akt are many and diverse. Of particular interest to osteoblast function we have shown that activation of Akt via CaSR, decreased oxidative stress-induced apoptosis, increased growth via activation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and induced canonical Wnt signaling via GSK3\textsubscript{b} inactivation. The activation of Akt itself occurs via two distinct pathways; PI3-kinase/PDK1-dependent phosphorylation at Thr308 and mTOR complex 2 (mTORC2)-dependent phosphorylation at Ser473 - signifying that mTOR complexes reside both upstream and downstream of Akt in bone cells. We have used human bone-derived osteoblasts as a model system to understand CaSR-dependent activation of Akt in relation to mTOR in a non-transfected, primary cell type. The major finding or point of emphasis of the present study is to provide a potential mechanism for CaSR-dependent mTORC2 activation in bone cells.
THE EXTRACELLULAR CALCIUM (Ca²⁺o)-SENSING RECEPTOR AND DISORDERS OF Ca²⁺o-SENSING

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The extracellular calcium (Ca²⁺o)-sensing receptor (CaSR) mediates the suppressive actions of Ca²⁺o on PTH secretion and parathyroid cellular proliferation. A characteristic feature of most forms of hypercalcemic hyperparathyroidism (HPT) is reduced sensitivity of PTH secretion to the inhibitory action of elevated levels of Ca²⁺o combined with parathyroid enlargement. Inherited diseases with resistance of the Ca²⁺o -sensing mechanism to Ca²⁺o have shown that this abnormality in parathyroid Ca²⁺o -sensing can occur at the level of the CaSR, downstream at Gα11, which couples the CaSR to activation of PLC, or at the sigma 1 subunit of the AP2 complex. In the majority of acquired forms of hypercalcemic HPT (e.g., primary, secondary/tertiary, lithium-induced HPT) as well as in hereditary forms of HPT that are not thought to directly affect the Ca²⁺o-sensing apparatus (e.g., HPT in MEN or HPT-JT), there is also reduced sensitivity of the Ca²⁺o-sensing mechanism to hypercalcemia. However, the underlying mechanism(s) is/are not well understood and are an area of active investigation. This presentation will review the mechanisms underlying abnormal Ca²⁺o-sensing both in conditions with known molecular mechanisms and in those that are less well understood. A conceptual framework will be developed that may help to frame outstanding issues in this area.
THE CALCIUM SENSING RECEPTOR IN THE NORMAL BREAST AND IN BREAST CANCER

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Expression of the CaSR gene is increased in the normal mammary gland during lactation and the receptor is found on the basolateral surface of alveolar epithelial cells. The demand for calcium associated with milk production stresses maternal calcium homeostasis and mothers adapt, in part, by resorbing bone to release skeletal calcium stores. We have found that activation of the CaSR increases transepithelial calcium transport into milk and inhibits secretion of parathyroid hormone-related protein (PTHrP) into the maternal circulation and milk. PTHrP from the breast circulates to increase bone resorption in the mothers and milk PTHrP inhibits calcium accrual in neonates. Therefore, expression of the CaSR allows the mammary gland to directly participate in maternal bone and calcium metabolism. In a classic feedback loop, mammary epithelial cells monitor the delivery calcium to the gland and adjust calcium usage and bone resorption to maintain a constant supply of calcium for milk production and to prevent maternal hypocalcemia. Interestingly, in breast cancer cells, the CaSR increases rather than decreases PTHrP production as a result of switching receptor coupling from Goi to Gos possibly by heterodimerizing with the GABA-B receptor 1. Consistent with these observations in vitro, we have found a positive correlation between CaSR and PTHrP mRNA and protein expression in two cohorts totaling over 800 patients with breast cancer. Expression of the CaSR in a series of 652 breast tumors demonstrated that it correlated with a more aggressive tumor phenotype and inversely with survival. In breast cancer cells, the CaSR appears to stimulate cell proliferation and to inhibit cell death though mechanisms involving intracrine actions of PTHrP. Finally, knocking out the CaSR in mammary epithelial cells slows tumor growth in the MMTV-PyMT transgenic model of breast cancer. Thus, our major findings are that the CaSR has important roles in both normal mammary physiology and breast cancer.
CaSR IN EPIDERMAL DIFFERENTIATION AND WOUND HEALING

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The epidermis is a stratified squamous epithelium composed of proliferating basal and differentiated suprabasal keratinocytes. A calcium gradient within the epidermis promotes the sequential differentiation of keratinocytes as they traverse the epidermis to form the permeability barrier of the stratum corneum. The CaSR regulates cell survival and keratinocyte differentiation by initiating the intracellular signaling events in response to extracellular calcium (Cao) via two major pathways. First, CaSR controls intracellular calcium (Cai) stores and Cai handling by interacting with modulators of Cai store and membrane ion channels. Second, CaSR, through physical interaction with filamin A, forms a signaling complex with Rho A GTPase and guanine exchange factor Trio to stimulate the formation of the E-cadherin/catenin complexes in the membrane. These complexes underlie intercellular adhesion and serve as a scaffold for recruiting and activating other signaling molecules critical for cell survival and differentiation. Inhibiting CaSR expression in vitro markedly increases apoptosis, suppresses Cai responses to Cao, and impairs cell differentiation by reducing Cai pools and blocking E-cadherin-mediated signaling. In vivo, the keratinocyte-specific CaSR null (EpidCaR/-) mice lose epidermal calcium gradient and manifest a reduction in differentiation markers and permeability barrier function in the epidermis. Furthermore, skin wounding triggers a substantial increase in CaSR expression, indicative of a role for the CaSR in wound healing. EpidCaR/- mice under dietary calcium restriction display a reduction in the innate immune response, pro-inflammatory cytokines, growth factors, and matrix metalloproteinases in wounded areas, and consequently, a delay in wound closure. Our studies demonstrate the importance of CaSR-mediated signaling in regulating epidermal differentiation, permeability barrier homeostasis, and wound repair.
EXTRACELLULAR CALCIUM IONS ACT AS A PRO-INFLAMMATORY DANGER SIGNAL

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At sites of chronic infection, arthritis or autoimmune inflammation, increased extracellular Ca2+ concentrations can be present. Monocytic cells express CaSR and GPRC6A, both of which can be activated by such increased Ca2+ concentrations. The signaling cascade triggered under those circumstances involves activation of phospholipase C and an intracellular calcium increase. The activated effector mechanism is the assembly of the NLRP3 inflammasome, a multi-protein complex containing activated Caspase 1. Subsequently, pro-inflammatory cytokines including IL-1β and IL-18 are secreted in high concentrations. In a murine model of acute inflammation, exogenous calcium amplifies the magnitude of the inflammatory response. This calcium effect is mediated in part by GPRC6A, since the increase in inflammation is significantly reduced in GPRC6A knockout mice. Similar effects are also exerted by endogenous calcium released from the calcium stores of necrotic cells. We propose, therefore, that pro-inflammatory effects of increased extracellular Ca2+ are likely to be relevant in vivo in situations of increased calcium concentrations or calcifications, and that the inhibition of such effects could be a beneficial therapeutic approach.
CALCIUM-SENSING RECEPTOR REGULATES NUMEROUS SIGNALING PATHWAYS IN NEURONS

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The search to identify an extracellular $[\text{Ca}^{2+}]_o$ sensor that could reduce the impact of physiological and pathological decreases in synaptic cleft $[\text{Ca}^{2+}]$ spurred interest in brain Calcium-sensing receptor (CaSR). This new interest has expanded with the recognition that some CaSR mutations cause epilepsy and that CaSR antagonists may be neuroprotective. We used direct patch-clamp recordings from small nerve terminals to show that CaSR stimulation inactivated a voltage-dependent, non-selective cation channel (NSCC) in the vast majority of nerve terminals in the cerebral cortex. We found that evoked excitatory synaptic transmission is enhanced in CaSR/- neurons and that CaSR activation impairs action potential-dependent release of glutamate. Thus, CaSR is positioned to respond to physiological decreases in $[\text{Ca}^{2+}]_o$, which substantially impair synaptic transmission. We also determined that CaSR activation enhances spontaneous release of both excitatory and inhibitory transmitters, and plays a role in regulating intrinsic excitability of neocortical neurons. The major finding or point of emphasis of the present study is that the CaSR has a complex role in the brain, and that further understanding of these signaling pathways is necessary to determine how CaSR signaling triggers seizure activity or reduces neuronal death. Research was supported by NIGMS.
COULD CALCILYTICS STALL ALZHEIMER’S DISEASE (AD) PROGRESSION?

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Extracellularly accruing Amyloid-β oligomers (Aβ-os) start AD in the aged lateral entorhinal cortex via: (i) the intranuclear migration and direct activation of the transcription of Aβ precursor protein (APP) and β-secretase/BACE1 genes, whose proteins acting with γ-secretase overproduce endogenous Aβ-os in both human astrocytes and neurons; (ii) the binding and activation of human astrocytes’ and neurons’ cell membrane CaSRs, the signalings of which hinder Aβ peptides cleavage by the proteasome and various proteases but favor the oversecretion via the Golgi/trans-Golgi network of the accruing endogenous Aβ-os; and (iii) an increased activity of GSK-3β, the main Tau protein kinase. Concurrently, Aβ/CaSR signaling elicits the overrelease of neurotoxic amounts of nitric oxide (NO) and vascular endothelial growth factor (VEGF)-A from the human adult astrocytes. Oversecreted Aβ-os spread, enter the nuclei and simultaneously bind the surface CaSRs of ever increasing numbers of adjacent astrocytes and neurons, thereby recruiting them to overproduce and oversecrete further bulks of Aβ-os. While the astrocytes survive the neurotoxic onslaught, the neurons start dying. Thus, via self-sustaining “contagious” cycles the neuropathology gradually spreads to the cognitive areas of cerebral cortex driving AD progression. However, we have shown that highly selective allosteric CaSR antagonists (“calcilytics”), like NPS 2143 and NPS 89626, totally suppress the GSK-3β overactivation and the Aβ-os, NO, and VEGF-A oversecretion from Aβ-os-exposed untransformed human cortical adult astrocytes and postnatal neurons cultured in vitro. Most important, calcilytics keep the neurons alive. Thus, the major findings of our preclinical studies show that (i) Aβ-os/CaSR signaling promotes the oversecretion of three main AD neurotoxins, i.e. Aβ-os, NO, and VEGF-A, and Tau protein phosphorylation too in both human neurons and astrocytes; and (ii) all these effects triggered by Aβ-os/CaSR are wholly suppressed by administering a calcylitic drug. Therefore, if given at an early enough stage, calcilytics could halt any further AD progression, preserve cognitive abilities, and thus save huge human, emotional, healthcare, and societal costs.
THE CALCIUM-SENSING RECEPTOR (CaSR) REGULATES BLOOD PRESSURE, VESSEL TONE AND CARDIAC FUNCTION

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The extracellular calcium-sensing receptor (CaSR) is expressed in blood vessels where its functions remain poorly understood. To better understand the physiological roles of the CaSR in the vasculature, we generated mice with CaSR gene ablation from cardiac and vascular smooth muscle cells (VSMC) by crossing exon 7 LoxP-CaSR mice with animals expressing Cre-recombinase under the control of the SM22α promoter. Cre-negative (wild-type, WT) and -positive (knock-out, KO) mice were comparable in size, fertility and life span. However, tail cuff and radiotelemetry experiments showed that blood pressures of KO animals were significantly reduced compared to WT by \textasciitilde 5\% (systolic) and \textasciitilde 9\% (diastolic). The observed hypotension was most pronounced during the active phase of the animals, and was insensitive to a high salt-supplemented diet and the NO synthase inhibitor, L-NAME. KCl- and phenylephrine-induced contractility was reduced in ex vivo isolated aortae and mesenteric arteries from KO animals compared with WT vessels. Exposure to increasing extracellular Ca2+ in concentrations (1-5 mM) evoked contraction followed by relaxation in WT, but relaxation only in KO aortae. KO animals exhibited bradycardia in vivo as well as reduced spontaneous activity in isolated hearts ex vivo and cultured cardiomyocyte-like cells. These studies indicate an unprecedented role for the CaSR in setting blood pressure levels, via amplification of contractile stimuli in blood vessels, and in the control of cardiac function, by directly contributing to cardiac pacing.
THE CALCIUM SENSING RECEPTOR PARADOX IN CANCER

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After 21 years of intensive research it is clear that the CaSR is expressed (almost) ubiquitously and regulates a plethora of different processes besides calcium homeostasis. Depending on the tissue where it is expressed, it can regulate hormone synthesis and secretion, gene expression, ion channel activity, inflammation, proliferation, differentiation, apoptosis, to name but a few. Altered CaSR signalling is associated with a number of pathophysiological states. In cancer, CaSR-mediated signalling may differ dependent on the type of cancer, stage, and grade. During malignant transformation different CaSR-dependent signalling pathways become activated than those activated in the respective normal tissues. In tissues, where the CaSR usually suppresses growth and promotes differentiation, the tumors will develop means to silence its expression, e.g. in parathyroid, colon tumors. It has been shown recently that the CaSR is expressed in differentiated neuroblastic tumors, but it is silenced in unfavorable neuroblastomas. In these tumors, dearth of CaSR expression results in loss of the growth suppressing effects of high levels of calcium. Activation of the receptor inhibits proliferation of these cancer cells, suggesting a tumor suppressor function for CaSR. In contrast, increased expression is observed in highly metastatic primary breast and prostate cancer cells, or in renal cell carcinomas that will metastasize to bone. In metastatic breast cancer cells the CaSR stimulates secretion of several chemotactic mediators with angiogenic properties. Furthermore, in breast cancer cells CaSR activates preferentially Gαs proteins and not Gαi, as in normal breast cells, resulting in increased production of parathyroid hormone-related peptide, which is a contributor to metastatic processes involving bone. In these settings, the CaSR seems to have an oncogenic role. Thus, in cancer the CaSR has paradoxical roles: it can either prevent, or promote tumorigenesis depending on the type of cancer. Changes in CaSR signalling during tumorigenesis seem to involve very different pathways, consistent with the different roles the CaSR plays in normal physiology.
CaSR AND VDR IN SKIN CANCER

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Vitamin D, via its active metabolite 1,25 dihydroxyvitamin D (1,25(OH)2D), and calcium are key regulators of epidermal differentiation, generally acting synergistically in part through the same pathways. 1,25(OH)2D exerts its effects primarily through genomic mechanisms mediated by the vitamin D receptor (VDR), a transcription factor and member of the nuclear hormone receptor family. Calcium acts through the calcium sensing receptor (CASR), a membrane bound member of the G protein coupled receptor family. We have developed mouse models in which the Vdr and Casr have been deleted specifically in keratinocytes (epidVdr-/- and epidCasr-/- respectively). The epidVdr-/- mouse loses hair follicle cycling after the initial developmental hair follicle cycle, a phenotype not observed in the epidCasr-/- mouse, but both lose features of terminal differentiation including the formation of the permeability barrier. In vitro studies with keratinocytes demonstrate that when production of either VDR or CASR is prevented, differentiation is markedly impaired including the ability to form the E-cadherin/catenin complex required for the differentiation process. In previous studies we and others showed that Vdr-/- mice (both global and epidermal specific) are predisposed to chemical or UVB induced tumor formation. Spontaneous tumors were not observed. Likewise, spontaneous tumors in epidCasr-/- mice have not been observed, but in mice with epidermal specific deletion of both Vdr and Casr (epidVdr-/- / epidCasr-/- [DKO]) tumor formation occurs spontaneously in a manner accelerated when the DKO mice are placed on a low calcium diet. To determine the mechanism(s) underlying the predisposition to tumor formation initially in Vdr-/- mice we examined both the hedgehog (HH) and wnt/b-catenin) pathways, pathways well known to have interacting roles in tumor formation in a variety of tissues including skin. We found that in the Vdr-/- mouse the HH pathway was overexpressed in the epidermis, and could be suppressed in normal skin explants with 1,25(OH)2D. Similarly, b-catenin transcriptional activity could be suppressed by 1,25(OH)2D and VDR in keratinocytes. The major point of this study is to explore the mechanisms by which VDR and CaSR interact to regulate the HH and b-catenin pathways and how the loss of such regulation leads to cancer.
THE CALCIUM-SENSING RECEPTOR IN NEUROBLASTOMA

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Neuroblastic tumors (NT) encompass a heterogeneous group of developmental malignancies that arise from the sympatheoadrenal lineage of neural crest cells, and include the neuroblastomas (NB), ganglioneuroblastomas (GNB) and ganglioneuromas (GN). Differentiated GNB and GN are usually benign, but the overall survival rates of metastatic NB remain below 40% and the prognosis of relapsed/refractory cases is uniformly fatal. Our group first described the expression of the calcium sensor receptor (CaSR) and parathyroid hormone-related protein (PTHrP) in differentiated NT, and their upregulation upon differentiation induction. We also reported that the CaSR gene is silenced by genetic and epigenetic events in undifferentiated, aggressive NB. In keeping with these findings, overexpression of the CaSR in NB cells reduces their proliferative rates and almost completely abolishes their tumorigenic capacities. Moreover, NB cells undergo apoptosis upon acute reactivation of the CaSR. We have also shown that an haplotype including three polymorphisms associated with moderate reduction of CaSR activity is an independent predictor of poor outcome in NT. Altogether, our published data support the hypothesis that the CaSR exerts tumor suppressor functions in this context. In keeping with this body of data, our present, unpublished results indicate that, in the developing neural tube, the CaSR is expressed in differentiated cells of the mantle zone, but not in highly proliferative, undifferentiated cells of the ventricular zone. Moreover, constitutively-active CaSR blocks neural crest cells migration through the somites. Finally, our current work also indicates that PTHrP is a growth factor required for NB cell survival.
TREATMENT WITH CINACALCET AND CARDIOVASCULAR OUTCOMES: ANALYSIS OF THE EVOLVE TRIAL

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The EVOLVE trial was a global, randomized, double-blind, placebo-controlled trial evaluating the effect of treatment with cinacalcet, a calcium-sensing receptor (CaSR) agonist, on the composite primary outcome of death or major CV event among 3883 prevalent hemodialysis patients. The unadjusted relative hazard for the primary outcome was not statistically significant however after accounting for imbalances in baseline characteristics of study participants the relative hazard was nominally statistically significant (0.88; 95% CI 0.79, 0.97). Beyond a difference in age between treatment groups, a significant age x treatment interaction was observed. To evaluate more thoroughly the effect of treatment with cinacalcet on cardiovascular outcomes, several additional pre-specified and post-hoc analyses have been conducted including evaluating the effects in older versus younger patients; atherosclerotic versus non-atherosclerotic events and the effect of FGF23 reduction on cardiovascular events. Each of these analyses will be briefly discussed. The major point of emphasis of the present review will be to highlight the cumulative body of data describing the effects of treatment with cinacalcet on cardiovascular outcomes in prevalent hemodialysis patients.
CINACALCET, FGF23/KLOTHO, AND PARATHYROID HYPERPLASIA

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Chronic kidney disease-mineral and bone disorder (CKD-MBD) is a very common and serious systemic complication in CKD patients, because it not only contributes to the development of bone disease, but also increases the risk of cardiovascular (CV) and all-cause mortality through soft-tissue and vascular calcification. Secondary hyperparathyroidism (SHPT), characterized by persistently elevated levels of parathyroid hormone (PTH), is one of the major abnormalities underlying CKD-MBD. With longer dialysis vintage, parathyroid hyperplasia develops in these patients forming nodular hyperplasia. Because three major negative feedback pathways on parathyroid function, i.e., vitamin D receptor, calcium and FGFR-klotho complex are down-regulated in these glands, such patients often become resistant to various therapies including vitamin D receptor activators (VDRAs). Cinacalcet is a calcimimetic agent that has been developed for the control of PTH secretion. It inhibits the secretion of PTH by increasing the sensitivity of the calcium-sensing receptors on the surface of parathyroid cells. In sharp contrast to VDRAs, cinacalcet suppresses PTH without causing hypercalcemia and/or hyperphosphatemia. Cinacalcet treatment has also been shown effective even in most severe patients with nodular hyperplasia. Since the introduction of cinacalcet in Japanese market, the number of surgical parathyroidectomy has significantly decreased. Our recent data suggested that cinacalcet induces apoptosis of hyperplastic parathyroid cells, leading to regression. In our cohort study (MBD-5D study), cinacalcet use was associated with reduced risk of cardiovascular event and death, as in EVOLVE study.
 PRIMARY HYPERPARATHYROIDISM: MEDICAL MANAGEMENT WITH CALCIMIMETIC THERAPY

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Parathyroid cells (PTCs) from glands removed from patients with primary hyperparathyroidism (PHPT) demonstrate multiple abnormalities: a shift to the right in their set-point for suppression of parathyroid hormone (PTH) secretion by high extracellular calcium concentrations ([Ca2+]e) or negligible suppressibility by high [Ca2+]e, reduced signaling by high [Ca2+]e, and decreased expression of the CaSR mRNA and protein. Studies have further shown that the degree of shift to the right in the Ca2+ set-point correlates with the level of CaSR expression in tissues removed from patients with PHPT. Thus, CaSR expression and functional abnormalities in secretory control are tightly linked in PHPT. Despite these abnormalities in CaSR expression and function, higher than physiologic [Ca2+]e can suppress PTH in patients with PHPT (1). The fact that the calcimimetic cinacalcet can shift signaling responses and the suppression of PTH secretion by high [Ca2+]e to the left in a dose-dependent manner (2) underscores the potential for such an agent to reduce PTH hypersecretion in PHPT. Initial studies showed that cinacalcet given orally to patients with mild PHPT reduced serum [Ca2+] into the normal range in 80-90% of patients accompanied by a lowering of steady-state PTH levels compared to placebo, and these effects persisted for up to 5 years of treatment (3). Serum phosphate levels also fell significantly, but 1,25-(OH)2 vitamin D levels did not change significantly. Biochemical markers of bone turnover rose modestly, however, bone mineral density remained stable without significant increases. Cinacalcet is also effective in patients who meet criteria for surgical management of PHPT but are unable to have surgery (4). This research shows the efficacy of treatment of PHPT with calcimimetic therapy.

AMG 416 is a synthetic peptide comprised of 7 D-amino acids linked to L-cysteine by a disulfide bond. It interacts with the extracellular domain of the calcium-sensing receptor (CaSR) and functions as an allosteric agonist thereby inhibiting parathyroid hormone (PTH) release. AMG 416 has been formulated for intravenous (IV) use, and its half-life is sufficiently long to permit thrice-weekly dosing to treat secondary hyperparathyroidism (sHPT) among those with chronic kidney disease (CKD) receiving hemodialysis. In adults with normal kidney function, IV doses of AMG 416 lower PTH levels within 1-2 hours in a dose-dependent manner, whereas blood ionized calcium (Ca) levels decrease more slowly reaching a nadir after 10-12 hours. Similar changes occur among subjects receiving hemodialysis. In a phase 2 study comprised of 12 weeks of dose titration followed by 40 weeks of dose adjustment in 37 hemodialysis patients with PTH >350 pg/mL and serum Ca >8.5 mg/dL at screening, mean (SD) PTH decreased from 862 (672) pg/mL at baseline to 444 (676), 305 (550), and 252 (131) pg/mL, respectively, at weeks 13 (n=32), 26 (n=25), and 52 (n=20). Four subjects (13%) had symptomatic hypocalcemia, and 3 (10%) had a serum Ca <7.5 mg/dL. Two large phase 3 placebo controlled clinical trials assessed the safety and efficacy of AMG 416 among hemodialysis patients with PTH >450 pg/mL and serum Ca >8.3 mg/dL. Both trials enrolled >500 subjects and included 26 weeks of treatment. Primary endpoints were the proportion of subjects with >30% decrease in PTH from baseline during the final 7 weeks of treatment which occurred in 75.3% and 74.0 %, respectively, of those given AMG 416 and in 9.6% and 8.3%, respectively, of those given placebo. The frequency of gastrointestinal side effects was similarly low in each treatment group. AMG 416 thus is effective in hemodialysis patients with sHPT, and it may obviate untoward effects that limit medication adherence and efficacy with currently available oral calcimimetic agents.
FAMILIAL HYPOCALCIURIC HYPERCALCEMIA (FHH) TYPES 1-3 (FHH1-3)

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The extracellular calcium (Cao2+)-sensing receptor (CaSR), a family C G-protein-coupled receptor (GPCR), regulates Cao2+ homeostasis by detecting alterations in Cao2+ concentrations and activating G-protein mediated signalling cascades, which modulate parathyroid hormone (PTH) secretion and urinary calcium excretion. The gene encoding the CaSR is located on chromosome 3q21.1, and mutations resulting in loss-of-function lead to familial hypocalciuric hypercalcemia (FHH), a lifelong disorder associated with mild-to-moderate elevations of serum calcium concentrations, normal or elevated PTH concentrations, and inappropriately low urinary calcium excretion. CaSR mutations are detected in ~65% of FHH patients, referred to as FHH type 1 (FHH1), and genetic linkage studies in other FHH kindreds revealed additional loci on chromosomes 19p and 19q13.3, designated FHH2 and FHH3, respectively, indicating genetic heterogeneity for FHH. FHH2 is due to mutations of G-protein subunit α11 (Gα11), encoded by GNA11, and in vitro expression of FHH2-associated Gα11 mutations were found to diminish the sensitivity of CaSR-expressing cells to Cao2+, consistent with a loss-of-function. FHH3 is due to loss-of-function mutations affecting adaptor protein-2 sigma subunit (AP2σ), encoded by AP2S1. AP2, a heterotetrameric complex, is involved in clathrin-mediated endocytosis and AP2σ mutations, which all affect the Arg15 residue that interacts with the dileucine motif of cargo proteins and comprise Arg15Cys, Arg15His and Arg15Leu, result in increased CaSR cell surface expression likely due to decreased CaSR internalisation. Such AP2σ mutations are found in >20% of FHH patients who do not have CaSR or Gα11 mutations. These studies have provided new insights into CaSR signaling and trafficking.
AUTOSOMAL DOMINANT
HYPOCALCEMIA
(HYPOPARATHYROIDISM)
TYPES 1 AND 2

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Autosomal dominant hypocalcemia (ADH) type 1, the most common genetic form of hypoparathyroidism, is caused by heterozygous activating mutations in the CaSR. About 90 different such mutations have been identified. Most are located in the extracellular domain and in a region encompassing transmembrane domains 6 and 7, including the 3rd extracellular loop of the CaSR. Gain-of-function mutations increase the sensitivity of the CaSR to extracellular calcium, shifting the calcium–PTH curve leftward. Consequently, PTH synthesis and secretion is suppressed at normal calcium concentrations. Clinically, patients present with hypocalcemia, which is often mild, hyperphosphatemia, low magnesium levels, and low or low-normal levels of PTH. Affected patients typically have increased urinary calcium excretion caused by a decrease in circulating PTH concentrations and the activation of the renal tubular CaSR in the thick ascending limb. Therefore, ADH carries a relatively high risk of nephrocalcinosis, especially when treated with calcium and active vitamin D; hence asymptomatic patients are typically not treated. Therapeutic attempts using calcilytics are currently under investigation in a phase 2 trial. Recently, five different heterozygous mutations in the alpha subunit of the G protein G11 (GNA11) have been identified in patients with ADH, and this novel form has been classified as ADH type 2. These mutations lead to a gain-of-function of G11, a key mediator of CaSR signaling. Therefore, the mechanism of hypocalcemia appears similar to that of activating mutations in the CaSR, namely an increase in the sensitivity of parathyroid cells to extracellular calcium, and in turn, lower PTH secretion. Similarities and potential differences between ADH types 1 and 2 will be discussed. One of the key unresolved questions is why the main phenotype of germline mutations in this ubiquitous G protein appears to affect only the parathyroid glands. Studies of activating mutations in the CaSR and gain-of-function mutations in GNA11 provide new insights into parathyroid and renal
physiology and disease. In the long run, these efforts could help define new drug targets.
THE CALCIUM RECEPTOR AS A THERAPEUTIC TARGET

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So far, the only drugs that have proven to be clinically useful are calcimimetics for treating hyperparathyroidism (HPT). The type II calcimimetic cinacalcet (an allosteric modulator of the CaR) is useful in treating secondary HPT, parathyroid cancer, and certain forms of primary HPT. The type I calcimimetic AMG 416 (an agonist of the CaR) has certain treatment advantages compared to cinacalcet and might be approved for treating secondary HPT in patients receiving renal replacement therapy. Calcimimetics might also be useful in treating certain hypophosphatemic disorders. Calcilytics have not been effective treatments for osteoporosis probably because they cannot raise plasma PTH to levels sufficient to cause an anabolic response in the skeleton. At least one calcilytic (NPSP795) has been repurposed and is now in clinical trials as a treatment for autosomal dominant hypocalcemia in patients with activating mutations of the CaR. Calcilytics might be useful in treating other disorders of systemic calcium homeostasis such as idiopathic hypercalciuria. The primary function of the calcium receptor (CaR) is to regulate systemic calcium homoeostasis and it is uncertain if calcimimetics and/or calcilytics will be useful in treating disorders not involving mineral metabolism. In parathyroid glands, the CaR is the dominant molecule regulating various cellular functions so it is the best target for regulating this gland. In non-calcemic tissues, the CaR does not appear to play such a dominant role and is just one of many molecular targets that might be therapeutically useful. Additionally, systemic administration of a drug targeting the CaR will affect blood levels of PTH and calcium. This effect might be minimized by developing tissue-selective calcimimetics but tissue-selective calcilytics are improbable. Another approach to minimize effects on mineral metabolism is to administer a calcimimetic or calcilytic topically or locally. These approaches might expand the therapeutic repertoire of calcimimetics and calcilytics to diseases beyond those of mineral metabolism.
CALCILYTICS AS NOVEL THERAPEUTIC FOR TREATING ASTHMA

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Airway hyperresponsiveness (AHR) and inflammation are the characteristic features asthma. Because plasma levels of polycations, such as poly-L-arginine, spermine and extracellular cationic protein (ECP), are increased during allergic asthma, we hypothesised that, during asthma, they might induce airway hyperresponsiveness and inflammation acting on the calcium-sensing receptor (CaSR). Immunofluorescence microscopy shows expression of CaSR in human and mouse airway epithelial and smooth muscle cells. In HEK293 cells stably expressing the human CaSR, ECP spermine, poly-L-arginine evoked concentration-dependent increase in intracellular Ca²⁺ concentration (measured by fura-2 fluorescence). This effect was blocked by the calcilytics NPS89636, NPS2143 and Calhex231, and was absent in empty vector-transfected HEK293 cells. Mice with targeted deletion of the CaSR from smooth muscle cells were generated by breeding exon 7, LoxP-CaSR mice with SM22α-Cre mice. In vitro, airway smooth muscle cells obtained from explants of intralobular bronchi explanted from floxed CaSR animals (KO) show decreased responses to CaSR agonists in comparison to cells isolated from wild type (WT) animals. Ex vivo, tension measurements of WT mouse intralobular bronchi show increased constriction to ACh after pre-treatment with CaSR agonists. In addition, bronchi from WT animals constricted, whilst bronchi from KO animals dilated to polycations. Spermine also enhanced ACh-induced bronchoconstriction in lung slices from WT but not KO mice, an effect which was prevented by calcilytic. In unrestrained, conscious mice, poly-L-arginine significantly augmented airway resistance, an effect which
was blunted by inhaled administration of calcilytic. In mouse models of allergic asthma (ovalbumin-sensitised/challenged and mixed allergen-sensitised), inhalation of NPS89636 suppressed both AHR and inflammation. Our findings provide a novel mechanism, which explains the pathogenesis of airway AHR and inflammation during asthma. Local administration of calcilytics might provide a novel therapeutic for the treatment of this disease, and possibly of other chronic inflammatory lung disorders.
DEFICIENCY OF EXTRACELLULAR CALCIUM-SENSING RECEPTOR (CaSR) ATTENUATES HYPOXIA-INDUCED PULMONARY VASOCONSTRICTION AND PULMONARY HYPERTENSION

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Pulmonary arterial hypertension (PAH) is a rare, progressive disease characterized by a persistent increase in pulmonary arterial pressure and pulmonary vascular resistance (PVR). Regardless of the initial pathogenic trigger, the major causes of elevated PVR in patients with PAH are chronic pulmonary vasoconstriction, pulmonary vascular remodeling, in situ thrombosis, and increased pulmonary vascular wall stiffness. An increase in cytosolic free Ca2+ in pulmonary arterial smooth muscle cells (PASMC) is not only a major trigger for pulmonary vasoconstriction but also an important stimulus for PASMC proliferation. In this study, our data showed that the extracellular calcium sensing receptor (CaSR) expression and function were both enhanced in PASMC from idiopathic PAH patients. Furthermore, CaSR and parathyroid hormone (PTH) double knockout mice (CaSR-/-PTH-/-, referred to as DK mice) were used to test the hypothesis that CaSR modulates pulmonary vascular tone and the response to hypoxia. Wild-type (WT) and DK mice displayed normal systemic and pulmonary arterial pressure under normoxic conditions. However, compared to WT mice, DK mice had significantly lower right ventricular systolic pressure (RVSP), less right ventricular hypertrophy by RV/(LV+S) ratios, and displayed less pulmonary vascular remodeling when exposed to hypoxia. For WT mice, extracellular Ca2+ caused an increase in [Ca2+]cyt in PASMC isolated from hypoxic mice. However, restoration of extracellular Ca2+ had little effect on [Ca2+]cyt in DK mice, as compared to [Ca2+]cyt in PASMC from WT mice. These data indicate that CaSR plays an important role in the development and progression of pulmonary vascular remodeling in mice with PH. CaSR deletions prevent the development of hypoxia-induced PAH in mice via its modulation of extracellular Ca2+ entry in PASMC,
suggesting that CaSR may be a novel therapeutic candidate for the treatment of PAH.
Pancreatic islets are key physiological regulators of blood glucose concentrations, and their malfunction is central to the pathogenesis of diabetes mellitus, which affects >330 million people worldwide. Islet G-protein coupled receptors (GPCRs) facilitate the effects of diverse extracellular stimuli, ranging from fatty acids to neurotransmitters and gut hormones, on α- and β-cells that secrete glucagon and insulin, respectively, and represent an exploitable target for the modulation of glucose homeostasis. The calcium-sensing receptor (CaSR) is one of the most abundantly expressed islet GPCRs, and has been previously demonstrated to promote insulin and glucagon secretion in ex vivo studies of isolated islets. However, the physiological relevance of the CaSR in islet function remains to be elucidated, and the Nuf mouse, which has hypocalcemia in association with an activating CaSR mutation (Leu723Gln), represents a model for examining the in vivo consequences of altered CaSR function on glucose homeostasis. Indeed, heterozygous and homozygous affected Nuf mice have significantly impaired glucose tolerance in association with reduced plasma insulin concentrations and diminished pancreatic islet mass. The contribution of systemic dyscalcemia to the glucose phenotype of Nuf mice has been evaluated by administering a calcilytic drug, which increases plasma calcium concentrations in wild-type and Nuf mice, but only influences glucose concentrations in Nuf mice. Thus, the impaired glucose tolerance of Nuf mice is rectifiable by calcilytic treatment, and these studies indicate the CaSR to be an in vivo determinant of plasma glucose concentrations and highlight a potential application of targeted CaSR compounds in modulating glucose metabolism.
Poster Presentation Abstracts
MIR-135B- AND MIR-146B-DEPENDENT SILENCING OF THE CALCIUM-SENSING RECEPTOR EXPRESSION IN COLORECTAL TUMORS

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Studies have shown that the calcium-sensing receptor (CaSR) partially mediates the antitumorigenic effects of calcium against colorectal cancer (CRC). The CaSR expression in colorectal tumors is often reduced. We have reported previously that loss of CaSR expression in CRC is caused in part by methylation of the CaSR promoter 2 and loss of acetylation of lysine 9 on histone 3. In this study, we investigated the impact of aberrant microRNA expression on loss of CaSR expression in CRC. A microarray study in two Caco-2 subclones (Caco2/AQ and Caco2/15) that have similar genetic background, but different CaSR expression levels identified 22 differentially expressed microRNAs that potentially target the CaSR. We validated these results by performing in vitro gain and loss of function studies with the top candidates: miR-9, miR-27a, miR-135b, and miR-146b. These studies revealed that modulation of miR-135b or miR-146b expression by mimicking or inhibiting their expression regulated CaSR protein levels in two different colon cancer cell lines: Caco2/AQ (with moderate endogenous CaSR expression) and HT29 (very low endogenous CaSR expression). Inhibition of miR-135b and miR-146b expression led to high CaSR levels and
significantly reduced proliferation, in line with the tumor suppressing nature of CaSR. In samples of colorectal tumors we observed overexpression of miR-135b and miR-146b, and this correlated inversely with the expression of the CaSR (miR-135b: $r=-0.684$, $p<0.001$ and miR-146b: $r=-0.448$, $p<0.001$), supporting our in vitro findings. In summary, we could demonstrate that miR-135b and miR-146b target the CaSR and reduce its expression in colorectal tumors.
IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL MUTATION IN THE HUMAN CALCIUM-SENSING RECEPTOR GENE RESPONSIBLE FOR AUTOSOMAL DOMINANT HYPOCALCEMIA.

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The human calcium homeostasis is very exact, responding to small fluctuations in the extracellular calcium-ion concentration. One of the keys is the calcium-sensing receptor (CaSR). Aim: To investigate whether there was an association between phenotype and genotype in a family clinically diagnosed with Autosomal Dominant Hypocalcemia (ADH). Patients and methods: The proband, a woman, her husband and their three children were all genotyped for mutation in the CaSR gene. Functional characterization of the specific mutant CaSR gene was evaluated by impacts of graded [Ca2+]o-stimulated signaling responses including: inositol phosphate hydrolysis and Ca2+i-release. The mutant receptor protein cell surface expression was evaluated by immunoblot analysis. The impacts were investigated in transient transfected HEK-293 cells. Results: The proband and two of the children were diagnosed with ADH, the DNA sequence analysis revealing a heterozygous missense mutation in codon 126 providing an amino acid change D126V. Linkage analysis showed inheritance of the mutant as autosomal dominant. The signaling response by [Ca2+]o-stimulation revealed a significantly higher basal level in IP3 accumulation with a left-shifted calcium set-point for D126V compared to WT CaSR. The maximum response (Emax) of D126V was lower but not significantly below that of WT. According to the Ca2+i-release the [Ca2+]o-stimulation has little effect, showing a normalization of the sensitivity, however a further decreases in Emax for D126V to 49% of WT Emax results. The lower Emax is probably due to a Ca2+i-stores depletion caused by the constitutive activation. The cell surface expression of D126V CaSR did not considerably differ from that
of the WT receptor. The major finding of the present study is identification of a novel constitutive activating mutation in a known hotspot of the CaSR gene. Additionally, D126V has a remarkably lesser impact on Ca2+i-release than on PIP2 hydrolysis, signifying that other pathway may contribute to CaSR-mediated Ca2+i-release.
FAMILIAL HYPOCALCIURIC HYPERCALCAEMIA TYPE 3 (FHH3)-ASSOCIATED MUTATIONS IN ADAPTOR PROTEIN-2 SIGMA SUBUNIT CAUSE REDUCED INTRACELLULAR CALCIUM SIGNALLING AND DELAYED OSCILLATION EVENTS

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Calcium binding to the calcium-sensing receptor (CaSR), a G-protein coupled receptor (GPCR), initiates activation of its associated G-proteins, leading to calcium release from intracellular stores, that is observed as oscillations at low extracellular calcium [Ca2+]o concentrations (maximally between 2-4mM), and elevated steady-state responses at higher [Ca2+]o concentrations (above 7.5mM). These oscillations are hypothesized to be an adaptive response to allow receptors to continue to respond to small calcium fluctuations during prolonged ligand exposure, without deleterious consequences to the cell. Recently, loss-of-function mutations of the sigma subunit of the clathrin-mediated endocytic adaptor protein-2, AP2σ, which affect CaSR function and signaling, have been identified in familial hypocalciuric hypercalcaemia type 3 (FHH3) patients. To date, all FHH3-associated mutations have been reported to involve Arg15 (R15) and to lead to one of three missense mutations: Arg15Cys (R15C), Arg15His (R15H) and Arg15Leu (R15L). We sought to investigate the effect of these FHH3-associated AP2σ mutations on CaSR signaling pathways by measuring intracellular calcium responses. Imaging was performed in Fura-2 loaded HEK293 cells stably expressing wild-type CaSR, and transiently transfected with red fluorescent protein (RFP)-tagged AP2σ-wild-type (WT) or FHH3-associated AP2σ-mutants R15C, R15H and R15L (n=30-35). AP2σ expressing cells were selected based on RFP expression, alternately illuminated with 340 and 380nm light, and 510nm emission wavelengths recorded in real-time in the presence of increasing concentrations of
[Ca2+]o. Peak responses of 340/380nm ratios were used to plot dose-response curves. Intracellular calcium responses increased in all AP2σ-expressing cells in response to the elevation of [Ca2+]o in a dose-dependent manner. However, responses in cells expressing AP2σ mutant proteins were significantly (p<0.05) reduced compared to AP2σ-WT expressing cells (EC50 (95% CI) – WT - 2.33 (1.91-2.63), R15C – 3.36 (2.94-3.78), R15H – 3.21 (2.89-3.69), R15L – 3.14 (2.72-3.72)). Further examination of calcium oscillations revealed that AP2σ mutations lead to three types of responses when compared to AP2σ-WT which were: 1) a reduction in total number of oscillating cells observed with mutants R15C and R15H; 2) similar distribution in responses to [Ca2+]o observed with the mutant R15H; and 3) altered distribution to WT that required a higher [Ca2+]o to result in maximal oscillations observed with the mutants R15C and R15L. This impairment in the intracellular calcium signaling events in cells expressing AP2σ-mutant proteins are consistent with a loss-of-function within the CaSR signaling pathway, and reflect the altered phenotypes observed in FHH3 patients.
CASR DEPENDENT G-PROTEIN ACTIVATION AND ITS DOWNSTREAM SIGNALLING IN THE COLON

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Intestine specific calcium-sensing receptor (CaSR) knockout has shown the importance of the CaSR in the colon. Therefore, we investigated the signals mediated by the CaSR in Caco2-15 cells, which expresses CaSR endogenously. In this study we assessed the impact of CaSR-mediated signaling on adenylate cyclase activation, kinase / transcription factor activation by phosphorylation and intracellular calcium level. Regulation of cyclic adenosine monophosphate (cAMP) expression was determined using alpha screen assay and intracellular calcium responses were measured using live cell imaging with Fluo4 calcium imaging dye. Phosphorylation was determined by traditional western blot, in-cell western or immunofluorescence. Targeting the CaSR with increasing concentrations of extracellular calcium led to adenylate cyclase activation. Interestingly, high calcium levels inhibited CREB phosphorylation in Caco2-15 cells while increasing phosphorylation of ERK1/2 and NFkB. Extracellular calcium was unable to evoke any intracellular calcium response in Caco2-15 cells suggesting that the up-regulation of the ERK1/2 phosphorylation is not via Gαq. In order to test whether the effect of calcium was mediated by the CaSR, we treated the cells with specific allosteric modulators of the CaSR (agonist NPS R-568 and antagonist NPS 2143). We further confirmed the role of the CaSR by overexpressing the wild type CaSR (CaSR-WT) in Caco2-15 cells, whereas the cells transfected with the empty vector (Emp) were used as controls. The positive allosteric modulator NPS R-568 as well as the overexpression of the CaSR further enhanced the effect of calcium, whereas the negative modulator NPS 2143 reduced these. Using G-protein specific inhibitors we assessed which G-protein subunits are mediating these signaling events. The major finding of this study is that extracellular calcium induces CaSR mediated activation of adenylate cyclase, ERK1/2 and NFkB and inhibits CREB phosphorylation in colon cancer cells. Identification of these intracellular pathways will enhance our understanding of the role of CaSR in the colon.
FUNCTIONAL AND STRUCTURAL STUDIES OF Ca\textsuperscript{2+}-SENSING RECEPTOR

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Both inactivating mutations of the Ca\textsuperscript{2+}-sensing receptor (CaSR), L173P and P221Q, markedly impaired the functional positive cooperativity of the receptor as reflected by [Ca\textsuperscript{2+}]o -induced [Ca\textsuperscript{2+}]i oscillations, inositol-1-phosphate (IP\textsubscript{1}) accumulation and extracellular signal-regulated kinases (ERK1/2) activity. In contrast, activating mutations, L173F and P221L, showed the opposite. Analysis of the dynamics of the mutants using computational simulation studies supported disruption in the correlated motions in the inactivating CaSR mutants, while enhanced motions in the activating mutants. L-Phe could modulate wild type CaSR in a heterotropic positive cooperative way, but had no effect on the L173P mutant. Both in silico analyses and studies in intact cells indicated that residue Leu173 exhibited impaired heterotropic cooperativity in the presence of L-Phe. We also purified the glycosylated ECD, containing either complex or high mannose N-glycan structures. Using various spectroscopic methods, we have shown that both protein variants bind Ca\textsuperscript{2+} with a K\textsubscript{d} of 3.0 -5.0 mM. The local conformational changes of the proteins induced by their interactions with Ca\textsuperscript{2+} were visualized by NMR. Studies using saturation transfer difference NMR suggested a direct interaction between the CaSR ECD and L-Phe. We further demonstrated that L-Phe increased the binding affinity of the CaSR ECD for Ca\textsuperscript{2+}. The major finding of the present study provides new insights into the mechanisms by which Ca\textsuperscript{2+} and amino acids regulate the CaSR and may pave the way for further exploration of the structural properties of CaSR and other members of family C of the GPCR superfamily.
BACTERIAL EXPRESSION AND PURIFICATION OF THE CALCIUM SENSING RECEPTOR’S EXTRACELLULAR DOMAIN

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The Calcium Sensing Receptor (CaSR) is a Class C G-protein coupled receptor (GPCR), structurally characterized by the presence of a large nutrient sensing N-terminal extracellular region consisting of a bilobed Venus Fly Trap (VFT) module and a cysteine-rich (CR) domain. Up to five primary binding sites for the endogenous ligand Ca2+ and a binding pocket for positive allosteric modulator L-phenylalanine have been identified in the VFT. In addition, the VFT module is known to be involved in receptor dimerization and activation. However, there are currently no available structures for CaSR’s structural domains. Therefore, the structural elucidation of the VFT module in the N-terminal extracellular region of the Calcium Sensing Receptor is anticipated to provide critical insight into mechanisms underlying the observed ligand promiscuity and receptor dimerization and cooperativity. Based on secondary structure predictions, disorder analysis and sequence alignments of CaSR across species and Class C GPCRs, six variants of CaSR extracellular domain (ECD) inserts were prepared and ligated into bacterial protein expression vectors pRSET, pGEX, pMal c2x and pMal p5x. Preliminary results from protein expression trials in E. coli Rosetta cells showed overexpression of the His-tagged CaSR ECD constructs confirmed by a western blot using anti-His antibody. However, interestingly most of the His-tagged protein appeared to be cleaved into two fragments although full-length protein and oligomers of the protein were visible in non-reducing conditions. Overexpression of GST tagged CaSR VFT proteins were also observed in Coomasie stained SDS-page gel and confirmed by a western blot using CaSR ADD polyclonal antibody. However, the protein does not seem to be soluble in any of the conditions tested. The low yield and solubility of the expressed proteins are limiting factors in further structural studies. Therefore, other expression systems including mammalian and insect cell systems are currently being investigated.
INTERACTION OF THE CALCIUM-SENSING RECEPTOR WITH TYPE 1 TASTE RECEPTORS

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The calcium-sensing receptor (CaSR) and taste receptors type 1 (T1Rs) are all class C G-protein coupled receptors that are co-expressed in GI-associated endocrine tissue including insulin-secreting pancreatic islet beta cells. The T1R family has three members, taste receptor type 1 member 1 (T1R1), taste receptor type 1 member 2 (T1R2) and taste receptor type 1 member 3 (T1R3). Although T1Rs form heterodimers of the type T1R1+T1R3, T1R2+T1R3 and at least one homodimer, T1R3+T1R3, it is not known whether the CaSR forms heterodimers with any of the T1Rs and, if so, their functional significance. The present study aimed to determine whether heterologous co-expression of the CaSR and one of the T1Rs in Chinese Hamster Ovarian (CHO) cells resulted in heterodimer formation and whether any novel receptors arising have distinctive functional properties. To this end, immunoprecipitation, using anti-FLAG beads (Sigma) and IPone assays have been conducted (CISBIO IPone kit). The results demonstrate that the CaSR preferentially forms heterodimers with T1R3. The calcium-sensing properties are comparable to that of the wild-type CaSR as reported by the IPone signaling pathway. The impacts of heterodimerization of the CaSR with the T1Rs on nutrient sensing and receptor-dependent biased signaling properties require investigation.
PROBING THE STRUCTURAL AND FUNCTIONAL STUDIES OF THE CALCIUM-SENSING RECEPTOR

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The calcium-sensing receptor (CaSR) belongs to the C class G-protein coupled receptor (GPCR) superfamily and is well known for its role in the regulation of the parathyroid hormone (PTH) and maintaining calcium homeostasis in humans. Mutations of the CaSR are associated with patients in familial hypocalciuric hypercalcemia (FHH), neonatal severe primary hyperparathyroidism (NSHPT) and autosomal dominant hypocalcemic hypercalciuria (ADHH). However, due to the large size of the extracellular domain (ECD), as well as its high degree of glycosylation, the CaSR has neither been visualized using protein crystallography nor NMR. In the present study, we utilized bacterial expression of the CaSR ECD, where a majority of the ligands interactions occur, to further verify the predicted Ca2+ binding sites. Circular dichroism, fluorescence spectroscopy, and gel electrophoresis have been applied to investigate how calcium modulates the structure of the receptor. In addition, we utilized real-time fluorescence microscopy to examine how calcium induced intracellular calcium signaling could be altered by mutations on Ca2+ binding sites in the CaSR transfected HEK 293 cells. The emphasis of this study is to confirm the proposed calcium binding sites within the CaSR expressed bacterially and in vitro. We have found that Ca2+ binding site mutations lead to a decrease or loss of Ca2+ binding capability as well as a sensitivity reduction in vitro.
HETERODIMERIZATION OF THE CASR AND THE GABA-BR1 ALTERS G-PROTEIN COUPLING IN ATT20 AND HEK 293 CELLS

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Our lab has shown that activation of the CaSR decreases PTHrP secretion in normal breast cells, but increases PTHrP secretion in breast cancer cells. The change in the response of PTHrP results from a switch in G-protein coupling. In normal cells, the CaSR couples to Gαi, which inhibits cAMP production and PTHrP production, while in malignant cells it couples to Gαs, which stimulates cAMP production and PTHrP production. The CaSR also couples to Gαs, activates cAMP production and stimulates PTHrP secretion in AtT-20 cells, (murine pituitary corticotroph-derived cells). Since the CaSR can heterodimerize with other GPCRs including the type B γ-aminobutyric acid 1 receptor (GABA-BR1), we measured GABA-R1 mRNA levels in AtT20 cells and breast cancer cells and found them to be elevated relative to normal mammary epithelial cells, leading us to hypothesize that heterodimerization of the CaSR and GABA-BR1 might favor coupling of the CaSR to Gαs. To test this idea, we knocked down GABA-BR1 gene expression in ATT-20 cells using 2 independent siRNAs. In cells treated with control siRNAs, activating the CaSR with calcium or gadolinium stimulated cAMP production. However, knocking down GABA-BR1 expression resulted in inhibition of cAMP production in response to calcium or gadolinium. As assessed by the 35S-GTPgS assay, activation of the CaSR led to recruitment of Gαs in the control cells but Gαi in the GABA-BR1 knockdown cells. HEK293 cells express low levels of both the CaSR and GABA-BR1, so, we transfected them with the CaSR alone, GABA-BR1 alone, or both receptors at a 1:1 ratio. Calcium and gadolinium had no effect on cAMP levels when HEK cells only expressed GABA-BR1. When only the CaSR was expressed, calcium or gadolinium decreased cAMP levels. However, when both receptors were expressed, calcium or gadolinium increased cAMP. Our major finding is that homodimerization of the CaSR favors coupling to Gαi while heterodimerization with GABA-BR1 favors CaSR coupling to Gαs. Current studies are examining whether this is the basis for altered PTHrP production in breast cancer cells.
CALCIUM-SENSING RECEPTOR INDUCES VASORELAXATIONS VIA NITRIC OXIDE GENERATION AND INTERMEDIATE-CONDUCTANCE CALCIUM-ACTIVATED POTTASSIUM CHANNELS

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CaSRs are expressed in the vasculature. The present work investigates how CaSR influences vascular tone. Methods: Mesenteric artery branches isolated from New Zealand White rabbits were mounted in a wire myograph and maintained in Krebs solution. Arteries were precontracted with 10µM methoxamine and cumulative additions of CaCl2 were added in the absence and presence of inhibitors tested. Isolated endothelial cells (ECs) were enzymatically dispersed from whole arteries. Results: CaSR expression at the EC plasma membrane was demonstrated by immunocytochemistry. Increasing [Ca2+]o 1.5-6mM induced vasorelaxations of precontracted arteries, abolished with the removal of the endothelium. Calhex-231, L-NAME (nitric oxide synthase inhibitor), KT5823 (protein kinase G inhibitor) and ODQ (guanylate cyclase inhibitor) all inhibited Ca2+o-induced vasorelaxations. TEA (non-specific K+ channel blocker), iberiotoxin (BKCa blocker) and 10µM linopirdine (Kv7 blocker) also attenuated the vasorelaxations. Adding Charybdotoxin (IKCa/BKCa blocker) together with iberiotoxin augmented the inhibition. Apamin (SKCa blocker), glibenclamide (KATP blocker) and indomethacin (COX inhibitor) had no effect. Nitric oxide production was observed in isolated ECs loaded with the fluorescent dye DAF-FM diacetate following CaSR stimulation, abolished by Calhex-231/L-NAME. Whole-cell perforated patch-clamp recordings observed CaSR-induced K+ currents sensitive to Calhex-231 and charybdotoxin but not apamin. Conclusions: CaSR mediates endothelium-dependent vasorelaxations via NO-GC-PKG signaling converging on VSMC BKCa and Kv7 channels. CaSR stimulation also activates endothelial IKCa to induce vasorelaxations. The major point of emphasis of this work is that endothelial CaSRs influence vascular tone by stimulating NO release and IKCa activity.
INTRINSIC PARATHYROID HORMONE SECRETION VIA PROSTANOID-DEPENDENT LOCAL MECHANISM

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The parathyroid provides continues surveillance of extracellular Ca2+ (Ca2+o) dependent on the calcium sensing receptor (CaSR) but the mechanism by which the CaSR controls PTH secretion is not well understood. We hypothesized that intrinsic PTH secretion is dependent on locally produced prostanoids and prostanoid receptors. Firstly, parathyroid cDNA microarray analyses were positive for the prostaglandin E receptor type-4 (EP4) and prostacyclin IP1 receptor as well as the synthase and prostacyclin synthase respectively. Secondly, the COX2 inhibitor NS398 (10-50 μM) acutely and reversibly suppressed intrinsic PTH secretion by approximately 50% from perfused human parathyroid cells stimulated to secrete by exposure to low Ca2+o (1.0 mM). Finally, selective inhibitors of the EP4 receptor L-161,982 (1-10 μM) and prostacyclin IP1 receptor (Ro1138452, 0.5-5 μM) acutely and reversibly suppressed PTH secretion. The results demonstrate that the intrinsic mechanism underlying PTH secretion is supported by the local production of prostanoids and autocrine or paracrine-dependent signaling via EP4 and IP1 receptors and indicate that the CaSR acts to disrupt the intrinsic mechanism.
KLOTHO AND CASR EXPRESSION IN PARATHYROID GLANDS IS CRITICAL TO REGULATE PTH SYNTHESIS AND PARATHYROID GLAND GROWTH

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The parathyroid gland (PTG) functions to maintain mineral ion homeostasis by detecting low serum calcium levels and secreting PTH. The calcium-sensing receptor (CaSR) is a key component in the homeostatic system to respond to serum calcium and regulate release of PTH. Klotho (KL) is also expressed in the PTG and is a co-factor for fibroblast growth factor 23 (FGF23), which acts on the PTG to suppress PTH as part of a regulatory feedback mechanism. To determine the specific role of Klotho in PTG and to test a possible interaction between CaSR and Klotho in proper PTH regulation, mice with parathyroid specific deletion of Klotho (PTH-KL KO), CaSR (PTH-CaSR KO) and both (PTH-DKO) were generated using Cre-LoxP recombination. PTH-KL KO mice have comparable body weight and life span compared to controls, but both PTH-CaSR KO and PTH-DKO mice were severely growth retarded. In particular, PTH-DKO mice exhibited a significantly decreased body weight and died earlier when compared to PTH-CaSR KO mice. Since PTH-CaSR KO and PTH-DKO died between 1 and 2 weeks of age, we compared serum biochemistries among all four genotypes at P10 to explore a potential interaction between Klotho and CaSR in parathyroid tissues to modulate mineral ion homeostasis. No differences were detected in serum Ca2+, Pi, PTH, iFGF23, and 1,25(OH)2D3 in PTH-KL KO compared to controls. Interestingly however, both PTH-CaSR KO and PTH-DKO mice displayed hypercalcemia, hypophosphatemia, and significantly increased serum PTH, iFGF23 and 1,25(OH)2D3 levels. Most notably however, PTH-DKO mice had further markedly increased serum PTH, iFGF23 and 1,25(OH)2D3 when compared to PTH-CaSR KO mice. Histological analysis showed that the PTG size in PTH-KL KO mice was similar to the one in controls, while PTH-CaSR KO mice showed
significantly larger glands. Interestingly, dual ablation of Klotho and CaSR in PTG resulted in a further enlargement of PTG size when compared to single PTH-CaSR KO mice. In particular, the parathyroid tissue of PTH-DKO mice exhibited more severe and disrupted morphological structures. Furthermore, we examined the proliferation of parathyroid cells using PCNA staining and detected increased proliferation in both PTH-CaSR KO and PTH-DKO mice. We then performed immunofluorescence to assess the PTH expression pattern. No significant changes in PTH protein expression were detected in PTH-KL KO mice, whereas CaSR ablation resulted in increased PTH expression. Surprisingly, in the extremely hyperplastic PTG of PTH-DKO mice most areas of altered tissue structure failed to express PTH protein. The more severe parathyroid gland phenotype in PTH-DKO mice indicates a possible role of Klotho in addition to the CaSR on suppressing PTH production and regulating cell differentiation. In summary, the major finding of the present study is that CaSR and Klotho play an essential and interrelated role in modulating PTH biosynthesis and parathyroid gland structure. The data indicate that Klotho has a suppressive function on PTH in the absence of CaSR and that loss of this function leads to detrimental consequences and earlier death.
CONTRIBUTION OF THE PARATHYROID CYP27B1 TO CIRCULATING 1,25(OH)2D LEVELS AND HYPERCALCEMIA


Renal 25-dihydroxyvitamin D 1-alpha-hydroxylase (Cyp27b1) is thought to be the major source of circulating 1,25-dihydroxyvitamin D3 (1,25D) and a major responder to increased parathyroid hormone (PTH) levels in producing hypercalcemia. To address whether the Cyp27b1 expressed in parathyroid cells (PTCs) also contributes to maintaining basal 1,25D levels and the development of hypercalcemia in primary hyperparathyroidism (HPT), we compared serum PTH (sPTH), Ca (sCa), and 1,25D (s1,25D) levels in mice with heterozygous or homozygous knockout (KO) of the calcium-sensing receptor (CaSR) (CaSR-Het or CaSR-Hom), homozygous Cyp27b1 KO (Cyp-KO), or both (CaSR-Het//Cyp-KO or CaSR-Hom//Cyp-KO) targeted specifically to PTCs. sPTH, sCa, and s1,25D levels were modestly but significantly increased in CaSR-Het vs. control (Cont) mice at 3 months of age, indicating a mild primary HPT phenotype. The CaSR-Hom mice died before 4 weeks of age with severe HPT and hypercalcemia. Interestingly, CaSR-Hom//Cyp-KO mice were viable at 3 months of age with reduced s1,25D (41.1±1.9 pM) and sCa levels (12.8±0.3 mg/dL) compared to CaSR-Hom mice (351±35 pM and 16.4±0.5 mg/dL, respectively), even though their sPTH levels are comparable, suggesting that hypercalcemia is the main cause for the early death of the latter mice. Similarly, ablating parathyroid Cyp27b1 significantly decreased sCa and s1,25D levels in CaSR-Het//Cyp-KO mice, despite the presence of higher sPTH levels compared to the CaSR-Het mice, suggesting the development of both primary and secondary HPT in the former mice. Cyp-KO mice developed only secondary HPT as indicated by reduced s1,25D and sCa levels and increased sPTH levels. The expression of renal Cyp27b1 RNA was increased by 40-300% in Cyp-KO, CaSR-Het//Cyp-KO, and CaSR-Hom//Cyp-KO mice, indicating insufficiency of kidney-derived enzyme production to restore s1,25D levels in the KO mice. The major finding of the present study proposes that parathyroid Cyp27b1 is a critical source of circulating 1,25D...
essential for regulating mineral metabolism in both basal and HPT states.
THE CALCIUM-SENSING RECEPTOR (CaSR) MEDIATES BIPHASIC Ca2+-DEPENDENT CONTROL OF CYP27B1 EXPRESSION

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Elevated extracellular Ca2+ (Ca2+o) suppresses 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) expression in the renal proximal tubule [1] but activates it in the parathyroid [2]. Thus Ca2+o has opposing effects on 1,25-dihydroxyvitamin D synthesis in different tissues. However, the underlying mechanisms remain unknown. The present study investigated the role of the CaSR in Ca2+o-mediated regulation of CYP27B1 expression using full length CYP27B1 (1501 bp) promoter-luciferase constructs transfected into control HEK-293 cells, or HEK-293 cells. As Ca2+o concentrations increased from 0.5-3.0mM, which was absent in control HEK-293 cells. As Ca2+o increased from 3.0-5.0mM, there was a pronounced reduction of expression, so that a biphasic Ca2+o-depedent response was observed from 0.5-6.5 mM with a peak at 3.0 mM Ca2+o. Interestingly, both responses were enhanced by cinacalcet (1.0 μM) abrogated CYP27B1 expression at the Ca2+o-concentrations tested. Inactivating response elements (AP1, CRE(p), CCAAT, and Sp1(p)) partially reduced Ca2+o-dependent activation at 3.0 mM Ca2+o. The major finding of the present study is that the CaSR mediates biphasic Ca2+-dependent control of CYP27B1 expression via multiple promoter response elements and signaling pathways.

THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR AS A MODULATOR OF INFLAMMATION: BUILDING A HYPOTHESIS

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The extracellular calcium-sensing receptor (CaSR) can be up-regulated by cytokines interleukin (IL)-1β and IL-6. These same cytokines also stimulate inflammatory bone resorption. Because the CaSR may modulate blood Ca concentration, the CaSR could serve to alter the intensity of the inflammatory response if blood Ca could be shown to affect any aspect of inflammation. Our work with a sheep model of burn injury demonstrated increased urinary excretion of C-telopeptide of type I collagen (CTx; Klein et al J Bone Miner Metab 2014) and hypocalcemia (Murphey et al Crit Care Med 2000) in the first 24h post-burn and a 50% up-regulation of the parathyroid CaSR by 48h post-burn (Murphey et al Crit Care Med 2000). These findings are similar to the elevated serum concentrations of IL-1β and IL-6, hypocalcemia and hypercalciuria encountered over the same time period in burned children, who by 6 wk post-burn have lost 7% of their lumbar spine bone mass. Thus the systemic inflammatory response results in cytokine-mediated bone resorption moving Ca from bone to blood and raising blood Ca concentration. Ability of CaSR to be up-regulated could alter blood Ca levels and buffer the increase in blood Ca by causing increased urinary excretion of Ca. Moreover, extracellular Ca has been shown in vitro to increase peripheral blood mononuclear cell production of certain chemokines, such as MCP-1 and RANTES (Olszak et al J Clin Invest 2000, Castro et al, abstract, J Bone Miner Res 2003), which help sustain an inflammatory response. Thus by lowering blood Ca concentration, the CaSR may reduce the intensity or duration of an inflammatory response. The major point of this paper is that the extracellular CaSR may serve as an adaptive response to systemic inflammation and by lowering blood Ca concentration may modulate the intensity or duration of an inflammatory process.
SYNERGISTIC EFFECTS OF A HIGH DIETARY CALCIUM AND EXOGENOUS PARATHYROID HORMONE IN PROMOTING OSTEOBLASTIC BONE FORMATION IN MICE

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We have investigated, in adult mice, whether calcium and exogenous PTH1-34 have synergistic effects on bone formation, and explored related mechanisms. Adult male mice were fed a normal diet or high calcium diet or combined high calcium diet and subcutaneously injected with PTH1-34 (80 μg/kg/day) for 4 weeks. BMD, trabecular bone volume, osteoblast number, alkaline phosphatase (ALP) and type I collagen positive areas, the expression levels of osteoblastic bone formation related genes and proteins were increased significantly in mice on the high calcium diet or treated with PTH, and even more dramatically in the combined dietary calcium with PTH treated mice. Osteoclast number and surface and ratio of RANKL/OPG were decreased in mice on the high calcium diet and were increased in PTH treated mice, not in the combined dietary calcium with PTH treated mice. Furthermore, third-passage osteoblasts were treated with high calcium (5 mM), PTH1-34 (10-8 M), or high calcium combined with PTH1-34. The osteoblast viability and ALP activity
were increased in either high calcium treated or PTH treated cultures and even more dramatically in the combined high calcium and PTH treated cultures with consistent up-regulation of osteoblast proliferation and differentiation related gene and protein expressions. These results indicate that dietary calcium and PTH play synergistic roles to promoting osteoblastic bone formation by stimulating osteoblast proliferation and differentiation.
THE EFFECT OF ALLOSTERIC MODULATORS OF CaSR IN ESOPHAGEAL EPITHELIAL CELLS

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CaSR, a G-protein coupled receptor, plays a role in glandular and fluid secretion in the gastrointestinal tract and regulates differentiation and proliferation of keratinocytes. CaSR is present in the esophageal epithelium, but its role in this tissue has not been defined yet. We investigated the effect of a CaSR agonist, cinacalcet (CCT), and antagonist, calhex (CHX), on cell growth and cell-cell junction proteins in primary cultures of porcine esophageal stratified squamous epithelium (SSE). We used MTT assay to monitor cell proliferation and immunohistochemistry (IHC) and Western blotting to monitor expression of CaSR and cell-cell adhesion molecules in long term experiments (24-48 hours). We used In-Cell Western to monitor changes in intracellular mediators in short term experiments (30 min to 4 hours). Our results indicate that SSE cells exposed to CCT (24-48 hours) changed from polygonal to spindle-shaped cells, a transformation that was proportional to CCT concentration. In CCT treated cells, staining with CaSR antibody showed increased expression of CaSR in the nuclear area. Furthermore, staining with E-cadherin and β-catenin antibodies showed redistribution of these proteins from the cell membrane to the cytoplasm. Immunoblot experiments showed reduced expression of β-catenin and increased expression of a proteolysis cleavage fragment of E-cadherin (Ecad/CFT2) in CCT treated cells. Long term CHX treatment increased the expression of p120-catenin, an adherens junction protein, and of RhoA, a GTPase involved in actin cytoskeleton remodeling. In-Cell Western assays at 30 min, 2 hours and 4 hours showed significant changes in PKC, phospho-PKC, Rac, phospho-Rac and PI3K in CCT and CHX treated cells. These changes were transient and time dependent. Our data indicate that long term CaSR activation causes formation of filopodia, decreases cellular β-catenin expression and increases expression of a proteolysis fragment of E-cadherin. The major findings of this study are that long term activation of CaSR disrupts the cadherin-catenin complex and relocates these proteins to the cytoplasm, possibly...
by dysregulation of Ca+2 influx into the cell, redistribution of CaSR and/or changes in intracellular mediators. These changes indicate an intricate and complex role for CaSR in esophageal cells.
CASR STIMULATES CI- AND SCFA-DEPENDENT BUT INHIBITS CFTR-DEPENDENT HCO3 SECRETION IN COLON

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Bicarbonate (HCO3-) secretion is a well-established physiologic process that is closely linked to overall fluid and electrolyte movement in the mammalian colon. These present studies show that extracellular calcium-sensing receptor (CaSR), a fundamental mechanism for sensing and regulating ionic and nutrient compositions of extracellular milieu in the small and large intestine, regulates HCO3- secretion. Basal and induced HCO3- secretory responses to CaSR agonists were determined by pH stat techniques used in conjunction with short-circuit current measurements in mucosa from rat distal colon mounted in Ussing chambers. R568, a specific CaSR activator, stimulated lumen Cl-- and short-chain fatty acid (SCFA)-dependent HCO3- secretion but inhibited cyclic nucleotide-activated glibenclamide-sensitive HCO3- secretion. Consequently, at physiological conditions (either at basal or during lumen acid challenge) when electroneutral CI-/ HCO3- and SCFA/HCO3-exchangers dominate, CaSR stimulates HCO3- secretion (via an effect on surface epithelial cells); in contrast, in experimental conditions that stimulate fluid and HCO3- secretion, e.g., when forskolin activates electrogenic CFTR-mediated HCO3- conductance, CaSR activation inhibits HCO3- secretion (via an effect on crypt epithelia). Similarly, activation of CaSR by R568 stimulated CI-- and SCFA- dependent HCO3-secretion and inhibited cAMP-dependent HCO3- secretion in colon mucosa of wild type mice; such effects were not observed in CaSR null mice. The major finding or point of emphasis of the present study is that intestinal HCO3- secretion may be fine-tuned by CaSR in accordance with nutrient availability and state of digestion and absorption. The ability of CaSR agonists to inhibit secretagogue-induced intestinal HCO3-secretion suggests that modulation of CaSR activity may provide a new approach to correcting volume and HCO3- deficits in diarrhea.
The main role of adipose tissue (AT) is to manage energy supply. Adipocytes store excess energy as triacylglycerols (TG), providing fatty acids when required. Dysfunctional AT is key in obesity-related cardiometabolic diseases development. Human adipose cells are among the numerous cell types that express the calcium sensing receptor (CaSR), with a possible role in obesity-related processes. We previously showed that CaSR activation in adipose cells is associated with inflammation and visceral adipogenesis, suggesting a dysfunctional effect. An important unexplored question is whether CaSR influences the adipocyte's ability to store TG, crucial to understand the possible whole-body consequences of CaSR-elicited AT dysfunction. The present work evaluated the effect of CaSR stimulation on TG accumulation and the expression of lipogenic enzymes in human adipocytes. In-vitro differentiated LS14 adipocytes were exposed to 1mM cinacalcet for 72h and TG content was evaluated by fluorimetry (Nile Red). Cinacalcet-treated adipocytes showed 30% lower TG content versus the untreated controls (p<0.005). The expression (mRNA) of lipid handling factors was evaluated in LS14 cells treated with cinacalcet 1mM for 24h. The calcimimetic decreased the lipogenesis-related enzymes glycerol-3-phosphate dehydrogenase (35%) and lipoprotein lipase (22%), whereas the de novo lipogenic enzyme fatty acid synthase and the master regulator of adipocyte phenotype peroxisome proliferator-activated receptor-γ remained unchanged. Despite the previously reported pro-adipogenic effect, our observations suggest a CaSR effect impairing adipocyte lipid accumulation, which -particularly under the cellular stress present in obesity-may result in ectopic fat deposition and organ toxicity. This
further supports our hypothesis of a negative impact of CaSR activation in AT, probably constituting a pharmacological target in obesity-related diseases. The major finding of the present study is that the activation of CaSR decreases TG content and lipogenic enzyme expression in differentiated adipocytes.
CALCIUM-SENSING RECEPTOR DISTRIBUTION IN MOUSE, RAT AND HUMAN KIDNEY

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Intra-renal distribution of the calcium-sensing receptor (CaSR) in the kidney is controversial, with discrepancies in the cellular localization, nephron segment and species expression patterns being reported. To clarify this issue, we set out to determine CaSR expression in mouse, rat and human kidney using in situ hybridization (ISH), immunohistochemistry (IHC) and proximity ligation assay (PLA). By ISH, CaSR mRNA was detected in thick ascending limb (TAL), distal tubule (DT) and collecting duct (CD), with TAL showing the highest levels of expression. CaSR protein distribution in the nephron was detected by IHC using antibodies raised against different CaSR epitopes [ADD (Thermo); N-terminal (Anaspec); C-terminal (custom made) and whole protein (Novus)], dual labeled with markers for TAL (Tamm-Horsfall), DT (thiazide-sensitive Na⁺-Cl⁻ cotransporter), and CD (aquaporin 2). The CaSR antibodies were selected from a panel of eight antibodies by assessing the immunofluorescence pattern in HEK293 cells stably transfected with the human CaSR. The specificity of these antibodies was further confirmed by western blotting in mouse, rat and human kidney tissue. CaSR immunoreactivity was detected in TAL, DT and CD, with higher expression observed in the TAL. In the proximal tubule (PT), identified by the brush border, lower levels of immunoreactivity were also detected. CaSR expression in the PT was confirmed by PLA, using ADD and C-terminus CaSR antibodies. No PLA signal was obtained when the primary antibodies were omitted or substituted by the Ig fractions. This study shows overlapping expression of CaSR mRNA and protein all along the nephron, from PT to CD, with the highest expression seen in the TAL. These findings clarify the expression pattern of CaSR in mouse, rat and human kidney, thus allowing the full physiological role of the receptor to be determined in this organ.
The extracellular Ca2+-sensing receptor (CaSR) renders cell responsiveness to changes in local and systemic Ca2+ concentrations. To determine whether CaSRs that are localized to growth hormone (GH)-releasing hormone (GHRH), pro-opiomelanocortin (POMC), and agouti-related protein (AGRP) neurons modulate neuroendocrine signals to control growth, metabolism, and skeletal development, we generated Neuron-CaSR-KO mice with CaSR gene knockout (KO) targeted to neurons. The Neuron-CaSR-KO mice were viable, but had smaller body sizes and weights and undermineralized skeletons. Reduced IGF1 and osteocalcin RNA expression in the CaSR-KO bones indicates impaired local IGF1 signaling and delayed osteoblast differentiation. In Neuron-CaSR-KO mice, serum GH and IGF1 levels were reduced to <20% of control mice along with reduced expression of GHRH and GH RNA by >50% in their hypothalami (Hyp) and pituitary gland (Pit), respectively, indicating a dysregulated Hyp-Pit-GH/IGF1 axis that could have contributed to stunted growth and skeletal phenotypes of the mice. Neuron-CaSR-KO mice also showed increased adiposity and serum leptin levels and decreased glucose tolerance along with reduced expression of POMC and increased AGRP expression in their hypothalamami. These data suggest that reduced sensitivity of POMC and AGRP neurons to leptin could have contributed to the increased adiposity. Additionally, Neuron-CaSR-KO mice showed reduced thyrotropin-releasing hormone and gonadotropin-releasing hormone expression in the Hyp and decreased thyrotropin, follicle-stimulating hormone, and luteinizing hormone expression in the Pit, indicating widely dysregulated hypothalamic functions and panhypopituitarism. Our data support that neuronal CaSRs play a critical role in integrating mineral, skeletal, and energy metabolism by controlling hypothalamic and other neuronal functions.
Voltage-gated sodium channels (VGSCs) are essential for action potential generation and propagation and are consequentially integral to intraneuronal signal transmission. Early physiological studies of VGSC function demonstrated an absence of modulation via second messenger pathways. However, VGSCs are strategically positioned to serve roles in mediating cellular plasticity. Cinacalcet, a positive allosteric modulator (PAM) of the calcium-sensing receptor (CaSR), produces a potent inhibition of VGSC current in cultured neocortical neurons. Cinacalcet-mediated blockade of VGSCs is substantially reduced by the GDP analog GDPβS. Furthermore, cinacalcet-induced inhibition of VGSC current persists in CaSR−/− neurons, implicating a CaSR-independent, though G-protein-dependent, mechanism of action. CaSR antagonists also demonstrated similar actions. The major finding or point of emphasis of the present study is that a PAM of the CaSR has off-target effects on VGSCs in neocortical neurons. Overall, these data indicate a signaling pathway from G-protein stimulation to changes in VGSC activity as a means of indirect modulation of neuronal excitability. Research was supported by NIGMS.
EXPRESSİON OF THE CALCIUM SENSING RECEPTOR IN ENDOTHELIUM REGULATES VASCULAR TONE THROUGHOUT CHANGES IN VASCULAR NO METABOLISM

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The Calcium Sensing Receptor (CaSR) is expressed in the blood vessels. Previous studies have shown a putative role of the vascular CaSR on the regulation of vascular tone, however the mechanism is still uncertain. To assess the ability of the receptor to modulate vascular tone, we used a tamoxifen-inducible mouse knock-out model with specific deletion of the CaSR from endothelial cells (IPDGFB-cre x loxP-CaSR). One week after induction, wire myography was performed on segments of aorta or mesenteric artery from knock-out (N≥4) and control (N≥4) animals. Knock-out aortae exhibited greater contraction in response to phenylephrine, which was abolished by incubation with the nitric oxide synthase inhibitor, L-NAME. In addition, knock-out aortae exhibited lower relaxation in response to acetylcholine than control aortae. No significant differences between knock-out and control animals were observed in mesenteric arteries exposed to all treatments. Blood pressure, measured by tail cuff, was comparable in knock-out and control animals. These findings suggest that short-term abrogation of the endothelial CaSR affects NO metabolism in aorta, thereby modifying vasoconstriction and dilatation, but is not sufficient to evoke significant changes in systemic blood pressure and does not result in modulation of vascular tone in mesenteric artery.
THE CALCIUM-SENSING RECEPTOR (CaSR) SUPPORTS THE GROWTH OF BREAST CANCER CELLS IN HIGH CALCIUM ENVIRONMENTS BY STIMULATING PARATHYROID HORMONE-RELATED PROTEIN (PTHrP) PRODUCTION

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Bone metastases from breast cancer cause pain, hypercalcemia, pathologic fractures and, ultimately, death. In order for breast cancer cells to grow in the bone microenvironment, they must adapt to high levels of extracellular calcium. Cells respond to extracellular calcium by activating the calcium-sensing receptor (CaSR), a G protein-coupled receptor that binds and signals in response to extracellular calcium. Previous reports have demonstrated that the CaSR is expressed in the lactating mammary gland and regulates parathyroid hormone-related protein (PTHrP) production and calcium transport into milk. In addition, the CaSR has been shown to be expressed in many breast cancer cell lines and its expression has been shown to be higher in bone metastases than in primary tumors in patients. Therefore, we studied the role of the CaSR in breast cancer cell behavior. First, we identified that activation of the CaSR with high extracellular calcium stimulated proliferation in human BT474 breast cancer cells. Knocking down expression of the CaSR inhibited proliferation in response to extracellular calcium. In addition, BT474 cells treated with the CaSR antagonist, NPS2143, showed decreased proliferation in a dose dependent manner. Furthermore, activation of the CaSR increased intracellular cyclic adenosine monophosphate (cAMP) levels and increased the production of PTHrP. Since PTHrP has been reported to increase proliferation in some breast cancer cell lines, we also knocked down PTHrP expression in BT474 cells, which reduced proliferation in response to increasing doses of calcium. Interestingly, BT474 cells treated with NPS2143 showed dramatic increase in apoptosis in response to increasing doses of extracellular calcium. Knocking down either the CaSR or PTHrP also dramatically
increased apoptosis in response to extracellular calcium in BT474 cells. These studies suggest that the CaSR promotes the survival and proliferation of breast cancer cells under calcium-rich conditions such as those found in the bone microenvironment, and that PTHrP acts as an important mediator of these effects. Ongoing studies are examining the consequences of altering CaSR expression on the growth of breast cancer cells in bone in vivo, but our preliminary studies suggest that the CaSR-PTHrP axis may be a useful therapeutic target to treat bone metastases in breast cancer.
THE CaSR: A NOVEL TARGET IN COLORECTAL CANCER PREVENTION

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The CaSR is expressed also in tissues not directly involved in calcium homeostasis like the colon. During colorectal tumorigenesis we found that CaSR expression is down-regulated, leading to the hypothesis that loss of CaSR provides growth advantage to transformed cells. Therefore we tested whether reintroducing the CaSR would reduce its carcinogenic potential. We stably transfected HT29 colorectal cancer (CRC) cells with the full length CaSR (HT29-CaSR) or an empty vector (HT29-EMP). Proliferation of HT29-CaSR cells significantly decreased whereas differentiation and apoptotic potential was significantly increased compared with HT29-EMP cells. These effects were more pronounced when the cells were treated with the calcimimetic, NPS R-568. Treatment with the calcilytic NPS 2143 could reverse these anti-neoplastic effects. Furthermore, HT29-CaSR cells had a more epithelial phenotype, reduced nuclear β-catenin levels and decreased invasive behavior compared with HT29-EMP cells. Therefore, we investigated expression of markers involved in epithelial to mesenchymal transition (EMT). Expression of EMT markers was significantly lower in HT29-CaSR cells, and the presence of the CaSR was able to further block upregulation of these factors in an EMT-inducing environment. Furthermore, we found that the presence of the CaSR was able to downregulate the cancer stem-cell like phenotype of these cells. To translate these findings in vivo we analyzed the colon of global CaSR knock out (CaSR/PTH double KO) and the intestine specific CaSR KO. Animals lacking CaSR had significantly lower expression of differentiation and apoptosis markers, whereas higher levels of proliferation and EMT-associated markers. Ex vivo, in a cohort of CRC patients, we found significant inverse correlations between CaSR expression and markers of proliferation and EMT, and positive correlations with differentiation/apoptosis markers. The major finding of this study is that the CaSR is a bona fide tumor suppressor in the colon. Loss of CaSR sensitivity leads to progression of this neoplastic disease. Our data support the rationale to develop pharmaceutical agents to restore colonic CaSR expression/ function during CRC.
CALCIUM-SENSING RECEPTOR (CaSR) REGULATES HUMAN FETAL LUNG DEVELOPMENT THROUGH THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE RECEPTOR (CFTR)

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Optimal postnatal respiratory function depends on successful completion of a complex development program that requires coordinated regulation of branching morphogenesis, vasculogenesis and fluid secretion in the fetal lung. These processes begin during the pseudoglandular stage (human: weeks 9 - 17, mouse: E11.5 - 16.5) and occur in a relatively hypercalcaemic environment (~1.7 vs. ~1.2 mM for a normal adult). Previously, we have established that this hypercalcemia suppresses lung branching and cellular proliferation, through activation of the calcium-sensing receptor (CaSR). Activation of the CaSR also partly modulates Cl--driven fluid secretion, measured electrophysiologically in lung explant cultures. This fluid is secreted from the lung epithelium by secondary active Cl- transport, resulting in a negative transepithelial potential difference, the magnitude of which reflects the rate of fluid secretion. Immunohistochemical localization has shown that a number of apical chloride channels, including the cystic fibrosis transmembrane conductance regulator (CFTR), are expressed in human fetal lungs. Inhibition of CFTR using Inhibitor-172 modulated Cl--driven fluid secretion in human fetal lungs, but only in CaSR-activating conditions (by either mimicking fetal hypercalcemia or through use of the calcimimetic NPS-R568). The major finding of this study is that fetal hypercalcemia is an important extrinsic factor in the normal development of the human lung. Furthermore, the CaSR plays an essential role in lung development.
through control of lung growth and fluid secretion through activation of the CFTR chloride channel.
THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR (CaSR) MEDIATES THE PROGRESSION OF OSTEOARTHRITIS BY MODULATING THE PROLIFERATION AND DIFFERENTIATION OF ARTICULAR CHONDROCYTES IN TEMPOROMANDIBULAR JOINT

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We investigated the role of CaSR in the development of OA and the underlying mechanisms. Through biomechanical dental stimulation with unilateral anterior cross-bite (UAC), OA was induced in the temporomandibular joint (TMJ) of Sprague Dawley rats and CartCre-ERTam(+/−)//CaSRflox/flox mice for 2-8 weeks. NPS2143 was injected into TMJ cavity of UAC-treated rats and CartCre-ERTam(+/−)//CaSRflox/flox mice, while the Tam was delivered to the latter mice through an IP route to determine the effect of inhibiting CaSR activity and expression on the development of OA. The actions of CaSR to stimulate downstream signaling molecules were elucidated in a chondrogenic ATDC5 cell line. Degeneration of TMJ cartilage was evident 2 weeks after UAC in both rats and control mice and worsened with time. In TMJ cartilage, the expression of parathyroid hormone-related protein (PTHrP) receptor (PPR) and cell proliferation were significantly decreased after 2 weeks of UAC and before the increased CaSR and Indian hedgehog (Ihh) expression and enhanced chondrocyte terminal differentiation that occurred 4 weeks after UAC. Inhibition of CaSR activity by NPS2143 in UAC-treated rats and ablation of CaSR expression in chondrocytes of the Tam-injected and UAC-treated CartCre-ERTam(+/−)//Casrflox/flox mice attenuated cartilage degeneration. Consistently, Ihh expression and terminal chondrocyte differentiation were reduced while PPR expression and chondrocyte proliferation was enhanced in those animals. Studies of ATDC5 cells suggested that CaSR activation may regulate chondrocyte proliferation and differentiation by modulating p38-PTHrP-PPR and ERK-Runx2-Ihh pathways, respectively.
The major finding or point of emphasis of the present study is that CaSR plays an important role in promoting UAC-induced OA by altering PPR and Ihh signaling to suppress proliferation and enhance the terminal differentiation of chondrocyte.
CASPALLOSTERIC MODULATORS
RECTIFY SIGNAL TRANSDUCTION
ABNORMALITIES ASSOCIATED WITH G-
PROTEIN ALPHA-11 (Gα11) MUTATIONS
CAUSING FAMILIAL HYPOCALCIURIC
HYPERCALCEMIA TYPE 2 (FHH2) AND
AUTOSOMAL DOMINANT
HYPOCALCEMIA TYPE 2 (ADH2)

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Germline loss- and gain-of-function mutations of G-protein alpha-11
(Gα11), which couples the CaSR to parathyroid and kidney signaling
pathways, give rise to familial hypocalciuric hypercalcemia type 2 (FHH2)
and autosomal dominant hypocalcemia type 2 (ADH2), respectively.
Cinacalcet and NPS-2143 are allosteric CaSR activators and inactivators,
respectively, that ameliorate signaling disturbances associated with
CaSR mutations, but their potential to treat FHH2 and ADH2 is unknown.
The aim of this study was to investigate the effectiveness of Cinacalcet
and NPS-2143 in rectifying intracellular Ca²⁺ alterations associated with
FHH2 and ADH2 causing Gα11 mutations, respectively. Wild-type and
mutant Gα11 proteins associated with FHH2 (L135Q and I200del) and
ADH2 (R181Q and F341L) were transiently transfected in HEK293 cells
stably expressing the CaSR. Alterations in intracellular Ca²⁺ were
measured by flow cytometry following exposure to extracellular Ca²⁺ and
allosteric modulators, and the half-maximal (EC₅₀) values
determined (n=6 experiments). This revealed that both FHH2-
causing Gα11 mutations led to ~30% increases in EC₅₀ values
(p<0.0001), whereas the ADH2-causing Gα11 mutations led to ~10%
decreases in EC₅₀ values (p<0.0001), when compared to wild type Gα11.
A dose-titration of Cinacalcet and NPS-2143 normalized the EC₅₀ values
of FHH2 and ADH2 associated Gα11 loss- and gain-of function
mutations, respectively, so that these values were not significantly
different from wild type Gα11. These findings demonstrate that CaSR allosteric modulators can successfully rectify signaling alterations due to FHH2 and ADH2-causing mutations and highlight the potential of these targeted CaSR compounds for the treatment of hypercalcemia and hypocalcemia caused by Gα11 mutations.
SKELETAL ANABOLISM BY CONCURRENTLY TARGETING THE PTH1R AND CaSR

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Intermittent parathyroid hormone (iPTH) is the only FDA-approved therapy that produces bone anabolism. A better understanding of the mechanisms underlying the actions and adverse effects of iPTH is required to devise strategies to enhance iPTH therapy. The objective of this study is to determine if the hypercalcemic effects of PTH is required to activate the extracellular calcium-sensing receptors (CaSR) in the osteoblast (OB) to produce osteoanabolic responses and if combined treatment with CaSR agonists enhances the osteoanabolic effects of iPTH. A pharmacological approach was taken to test whether activating CaSRs in OBs by co-injecting a calcimimetic enhances osteoanabolism of iPTH without producing hypercalcemia. Injections of PTH(1-34) produced hypercalcemia in mice, while injections of calcimimetic produced hypocalcemia 3 hrs after injections. In support of our hypothesis, co-injections with calcimimetic normalized the Ca2+-elevating effects of PTH (p<0.01). Skeletal analyses by μCT showed that PTH alone increased trabecular bone mass and thickness by ≈8% (p<0.05) in distal femurs when compared to vehicle-injected controls. When calcimimetics were co-injected with PTH(1-34), there were robust increases in trabecular bone mass (≈21%, p<0.05) and in thickness (≈17%, p<0.05). Co-injection of calcimimetic with PTH also significantly increased cortical total volume, bone volume and thickness by 8-10% (p<0.01). Furthermore, a loss-of-function approach using mice with their CaSR conditionally knocked out in OBs showed that ablation of CaSRs early in the OB lineage completely abrogated osteoanabolism induced by the combined PTH/calcimimetic treatment, supporting our hypothesis that OB CaSRs play an essential role in mediating skeletal responses to the treatment. The major finding or point of emphasis of this present study is the revelation of novel synergistic actions of iPTH and calcium in producing skeletal anabolism, which may establish preclinical regimens to restore osteoporotic skeleton and augment healing of bone injuries.
Chronic pro-inflammatory lung disorders, such as asthma and chronic obstructive pulmonary disease (COPD), affect over 0.5 billion people worldwide. They are characterized by airway hyperresponsiveness (AHR) and inflammation that have been associated with increase in sputum concentrations of certain polycations. Because certain polycations are agonists of the extracellular calcium-sensing receptor (CaSR), we hypothesized that calcilytics may prevent AHR and inflammation in asthma and COPD. Immunofluorescence showed expression of CaSR in human and mouse airway epithelial and smooth muscle cells. In HEK293 cells stably expressing the human CaSR, the polycations spermine, poly-L-arginine, and eosinophil cationic protein evoked concentration-dependent increases in intracellular Ca2+ concentration. This effect was absent in empty vector-transfected HEK293 cells and was prevented by the calcilytics, NPS89636, NPS2143 and calhex. Inhalation of poly-L-arginine significantly augmented AHR in unrestrained, conscious mice, whilst NPS89636 significantly diminished this effect. In a murine asthma model in vivo, the ovalbumin (OVA)-sensitized, OVA-challenged Balb/C mouse, inhalation of NPS89636 dose-dependently suppressed both AHR and inflammatory cell infiltration in the bronchoalveolar lavage fluid to an extent which was of comparable magnitude to the current standard of care, fluticasone. Inhalation of NPS89636 also significantly suppressed AHR and inflammatory cell infiltration into the lungs of LPS-treated Guinea pigs, an established preclinical COPD model. Together, these studies suggest that locally delivered calcilytics might provide a novel therapeutic strategy to combat AHR and inflammation during inflammatory lung disease.
SPECIFIC INTERACTIONS OF CALCIUM-SENSING RECEPTORS (CaSRs) WITH SOLUBLE AMYLOID-β PEPTIDES—A STUDY USING CULTURED CORTICAL NORMOFUNCTIONING ADULT HUMAN ASTROCYTES

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The CaSR is expressed by all kinds of cells residing within the central nervous system, including the astroglia. CaSR’s functions in the neural cells are complex and at present little understood. Recently, we showed that exposing cortical normofunctioning adult human astrocytes (NAHAs) to 2-to-20 μM exogenous fibrillar or soluble amyloid (A)β25-35 or Aβ42, the initial toxic driver of Alzheimer’s disease (AD), triggered the overproduction and overrelease of three AD-related neurotoxins, i.e. endogenous Aβ42, nitric oxide (NO) and vascular endothelial growth factor (VEGF)-A. We also demonstrated that the same doses of exogenous Aβ25-35 elicited in cortical postnatal human HCN-1A neurons an endogenous Aβ42 oversecretion followed by a progressive apoptotic cell death. All these effects brought about by the Aβ peptides could be wholly suppressed in either cell type by adding an allosteric CaSR antagonist (calcilytic) like NPS 2143, suggesting that Aβ peptides bind the CaSR and activate its intracellular signaling pathways. Therefore, we have surmised that Aβ/CaSR signaling at the level of both cortical neurons and astrocytes plays a crucial role in the self-induced spreading of the neuropathology and hence in AD promotion [1,2]. In the present study, we address the question of the specificity of the Aβ/CaSR interaction at the plasma membrane of the NAHAs and analyze the initial events occurring to the just formed Aβ/CaSR protein complexes. As a first step, by using classical immuno-fluorescence and colocalization methods we demonstrate the occurrence of a direct interaction between exogenous soluble biotin-labeled Aβ25-35 and the CaSRs at the plasma membrane of the NAHAs. Next, by means of the in situ proximity ligation assay (isPLA) [3], a method allowing to unambiguously image chosen protein–protein interactions within a maximum range of 30 nm, we show the formation of highly specific
complexes between Aβ25-35 and the CaSRs. Then, through a quantitative analysis of these forming Aβ25-35/CaSR complexes we show that their time-related kinetics at the plasma membrane level of the NAHAs are significantly affected by the addition of the calcilytic NPS 2143. Finally, we preliminarily show that at the plasma membrane the Aβ25-35/CaSR complexes form patches, an event that precedes their endocytosis [4,5]. In conclusion, the major finding of this study is the establishment of highly specific bonding interactions between exogenous soluble Aβ25-35 and the plasma membrane CaSRs mediating the activation of CaSR’s intracellular signaling pathways leading to the subsequent excess release of AD-promoting neurotoxins like Aβ42, NO, and VEGF-A. Moreover, the eventual endocytosis of the soluble Aβ25-35/CaSR complexes raises the need of further investigations aimed at clarifying the respective fates of the engulfed CaSRs and Aβs.

A NOVEL CASR MUTATION IN A TUNISIAN FHHH/NSHPT FAMILY ASSOCIATED WITH MENTAL RETARDATION

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The calcium-sensing receptor (CASR), a plasma membrane G-protein coupled receptor, is expressed in parathyroid gland and kidney, and controls systemic calcium homeostasis. Inactivating CASR mutations have previously been identified in patients with familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT). The aim of the present study is to determine the underlying molecular defect of FHH/NSHPT disease in a consanguineous Tunisian family. Mutation screening was carried out using RFLP-PCR and direct sequencing. We found that the proband is homozygous for a novel 15 bp deletion in the exon 7 (c.1952_1966del) confirming the diagnosis of NSHPT. All the FHH members were found to be heterozygous for the novel detected mutation. The mutation, p.S651_L655del, leads to the deletion of 5 codons in the second trans-membrane domain of the CASR which is thought to be involved in the processes of ligand induced signaling. The major finding of the present study is that the novel inactivating mutation was associated with the evidence of mental retardation in the FHH carriers. These results provide additional support for the implication of CASR gene in the FHH/NSHPT pathogenesis.
CHANGES IN PARATHYROID HYPERPLASIA IN CINACALCET-TREATED RENAL TRANSPLANT PATIENTS: SUBGROUP ANALYSIS OF A PROSPECTIVE SELF-CONTROLLED STUDY ON THE LONG-TERM USE OF CINACALCET FOR POST-TRANSPLANT HYPERCALCEMIC HYPERPARATHYROIDISM

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Cinacalcet mediates regression of parathyroid hyperplasia in hemodialysis patients with secondary hyperparathyroidism (HPT). Whether this occurs also in kidney transplant recipients (KTRs) with persistent hypercalcemic HPT, is unknown and was therefore investigated in a subgroup analysis of a prospective open-label self-controlled study on the long-term use of cinacalcet for post-transplant hypercalcemic HPT. In this study, we assessed the effects of cinacalcet on biochemical parameters of mineral metabolism in 44 stable KTR with hypercalcemic HPT by comparing pre- and post-treatment periods by summary statistics. We considered all repeated measurements of the relevant parameters between the date of initial hypercalcemia and cinacalcet initiation for the pre-treatment period, and all measurements up to 4 years after treatment start for the post-treatment period. In a subgroup of 13 KTRs, parathyroid ultrasound examinations were consecutively performed by one single experienced sonographer and gland volume and maximum diameter was studied over time. Four additional KTRs with hypercalcemic HPT who declined to take cinacalcet but had consecutive parathyroid ultrasound examinations, served as controls. In the subgroup of 13 cinacalcet-treated KTRs, parathyroid volume decreased from 0.27 (0.19-0.44) cm³ to 0.18 (0.11-0.39) cm³ after 23 (16-27) months. The maximum diameter remained stable. In the 4 controls, parathyroid gland volume increased from 0.27 (0.11-0.49)
cm3 to 0.38 (0.19-0.81) cm3 after 24 (9-27) months. In the overall cohort of 44 KTRs, cinacalcet was initiated after 1.8 (0.8-4.7) years post-transplant and was maintained for 6.2 (3.9-7.6) years. Cinacalcet decreased total serum calcium (sCa) and intact parathyroid hormone (iPTH) levels with a mean difference (95% CI) between pre- and post-treatment periods of -0.30 (-0.34 to -0.26) mmol/l for sCa and -79 (-103 to -55) pg/ml for iPTH (p<0.001 for both). Serum phosphate (sPh) and renal tubular reabsorption of phosphate to glomerular filtration rate (TmP/GFR) simultaneously increased after cinacalcet initiation reaching a plateau in the lower normal range after one year (sPh 0.19 (0.15-0.23) mmol/l; TmP/GFR 0.20 (0.16-0.23) mmol/l, p<0.001 for both). The key finding of the study is that in KTRs with hypercalcemic HPT cinacalcet not only improved biochemical parameters of HPT in the long-term but may also promote regression of parathyroid hyperplasia.
INVESTIGATION OF THE CALCIUM-SENSING RECEPTOR EXPRESSION IN MONOCYTES ISOLATED FROM SYNOVIAL FLUIDS

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Given the influence of pro-inflammatory cytokines on calcium-sensing receptor (CaSR) expression, we assessed CaSR expression in monocytes isolated from synovial fluid of patients with different types of rheumatisms and explored whether CaSR expression was related to the inflammatory nature of the synovial fluid. Pilot, cross-sectional, monocentric study in which were included all patients who presented with an articular effusion in the rheumatology ward. Surface and total CaSR expressions in monocytes isolated from synovial fluid and blood were assessed by flow cytometry analysis. U937 cells were cultured during 24 hours in presence of cell-free synovial fluids in order to specify the influence of different synovial fluids on CaSR expression in vitro. Forty one patients were included: osteoarthritis (n=10), microcrystalline rheumatisms (n=10), rheumatoid arthritis (n=12) and other inflammatory rheumatisms (n=9). In monocytes isolated from synovial fluid, the measure of CaSR expression (surface and total) shows that local pathological conditions influence CaSR expression. Indeed, a significant decrease of CaSR expression is observed between the osteoarthritis group and the 3 other groups with inflammatory rheumatisms and also between the microcrystalline rheumatism group and the rheumatoid arthritis group. Accordingly, CaSR expression in monocytes isolated from peripheral blood of the same patients was shown to be significantly decreased in rheumatoid arthritis patients compared to osteoarthritis. However, in circulating monocytes no other significant difference in CaSR expression were observed between groups, suggesting that CaSR expression in monocytes isolated from synovial fluid could be a marker of interest in rheumatic diseases. Confirming these data, CaSR expression was shown to be increased in a
dose dependent manner in vitro when U937 cells were incubated with synovial fluid from osteoarthritis patients. This effect was significantly lowered when “inflammatory” synovial fluids were used. As circulating monocytes, monocytes isolated from synovial fluid express CaSR. However, at the individual level, there are some differences in CaSR expression in monocytes according to their environment of isolation. This can be explained in one hand by the influence of synovial fluid on CaSR expression and on the other hand by the recruitment of a specific sub-population of monocytes in the synovial fluid. Further experiments are needed to address these questions. Together, our results indicate that CaSR expression measured in monocytes isolated from synovial fluid is influenced by extracellular microenvironment and suggest that it could be used as a complementary biomarker in some difficult diagnosis to differentiate the pathological conditions responsible for articular effusion.
POSSIBLE IMPLICATION OF THE EGF LIGAND/EGFR SIGNALLING PATHWAY IN CASR OVEREXPRESSING BREAST CANCER CELLS OSTEOLYTIC POTENTIAL

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The Calcium sensing receptor (CaSR) is a G-protein coupled receptor, crucial for the feedback regulation of the extracellular calcium homeostasis. Interestingly, in breast cancer the CaSR was found to be most abundantly expressed in patients that had already developed bone metastases, suggesting a potential role of the CaSR in bone metastasis development. Confirming this hypothesis, we demonstrated that after 14 days osteolytic lesions were more pronounced in mice that had been injected intratibially with MDA-MB-231 breast cancer cells overexpressing the wildtype form of the CaSR as compared with mice that had been injected with breast cancer cells overexpressing the dominant negative form (R185Q) of the CaSR (p=0.017) or the empty vector (p=0.026).

The aim of our study was therefore to better understand differences which exist between those cell lines at cellular and molecular levels, explaining their osteolytic aggressiveness. For our studies, we used MDA-MB-231 breast cancer cells overexpressing either a wildtype (CaSR-WT) or dominant negative form (CaSR-DN) of the CaSR. All the results were compared with cells transfected with the empty vector (zeocin resistant pcDNA3.1). A transcriptomic analysis of mRNA isolated from CaSR-WT versus EV MDA-MB-231 cells was performed. Over the 44000 mRNA assessed, only 40 were shown to be modulated in their expression. To name a few of them, BMP-1, FGF-13, VEGF-A, and epiregulin (EREG) appear up-regulated, while COL9A1, GRB14 and LRP4 were downregulated. Due to the role played by EGFR in cancer, specific
attention was paid to EREG which is an epidermal growth factor related to the EGF-like ligands. Quantitative RT-PCR analysis targeting the epieregulin mRNA confirms that CaSR-WT cells express 2.5 time more epieregulin gene than EV transfected cells. Interestingly, no significant difference was observed comparing EV and CaSR-DN cells. Furthermore, evidence was obtained that calcium ranging from 1.8 to 5 mM, significantly increases in a dose dependent manner the EREG expression in CaSR-WT cells. This last, reaches a 4 fold increase in cells exposed during 24 h to 5 mM calcium. No significant difference was shown when EV and CaSR-DN cells were exposed to calcium during 24 h.

The major finding or point of emphasis of the present study is that breast cancer cells overexpressing the CaSR may be more prone to induce bone loss, promoting the overexpression and secretion of Epieregulin, and the consequent activation of its mediated signaling pathway.
THE CALCIUM-SENSING RECEPTOR MEDIATES BONE TURNOVER IN MICE: MICROARRAY ANALYSIS IN ADULT LUMBAR VERTEBRA

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Our previous study demonstrated that parathyroid hormone (PTH) deficiency could result in osteoporosis before weaning and increase bone mineral content after adulthood. In this study, we confirmed the bone volume of vertebra was increased and proliferation of BMSCs was decreased in adult PTH−/− mice compared with their PTH−/−CaSR−/− littermates. To explore these issues, we collected vertebrae for microarray from the three genotypes mice at 12-week-old. Some up-regulation or down-regulation at mRNA and protein levels was confirmed by real-time RT-PCR and Western blots. Results revealed that the expression levels of molecules in the osteogenesis including PTHrP, p21, Wnt3a and Cyp27b1 were up-regulated, whereas suppressor of RANKL a crucial regulator of bone resorption was down-regulated in PTH−/− mice. To determine the effect of the PTH on bone metabolism and turnover, recombinant parathyroid hormone (PTH1–34) was performed in PTH−/−CaSR−/− mice and PTH−/− mice and WT controls for 4 weeks starting from 12 week of age. In littermates receiving vehicle, osteoblast number, ALP-positive area, type 1 collagen-positive area, and the expression of bone-formation-related genes, all were reduced significantly in PTH−/− mice compared with WT mice and were decreased even more dramatically in PTH−/−CaSR−/− mice. In addition, the tartrate-resistant acid phosphatase-positive (TRAP) areas, the ratio of RANKL/OPG were significantly reduced in PTH−/− mice and less significantly in PTH−/−CaSR−/− mice compared with WT mice. These parameters also were increased in WT, PTH−/−, and PTH−/−CaSR−/− mice following exogenous PTH treatment. PTH−/−CaSR−/− mice characterized by hyporesponsiveness to the metabolic effect of PTH1–
The result showed that deletion of CaSR can reverse the effect of PTH deficiency-induced inhibition of the bone turnover. In contrast, CaSR mediated anabolic effect of PTH. In conclusion, our results indicate that the CaSR mediates alterations in bone turnover in response to changes in PTH and PTHrP-stimulated bone metabolism in adult mice.
ASBMR Young Investigator Travel Award Winners

1. Abhishek Aggarwal: THE CaSR: A NOVEL TARGET IN COLORECTAL CANCER PREVENTION.


3. Yi Fan: KLOTHO AND CASR EXPRESSION IN PARATHYROID GLANDS IS CRITICAL TO REGULATE PTH SYNTHESIS AND PARATHYROID GLAND GROWTH.

4. Caroline Gorvin: FAMILIAL HYPOCALCIURIC HYPERCALCEMIA TYPE 3 (FHH3)-ASSOCIATED MUTATIONS IN ADAPTORPROTEIN-2 SIGMA SUBUNIT CAUSE REDUCED INTRACELLULAR CALCIUM SIGNALLING AND DELAYED OSCILLATION EVENTS

5. Amanda Herberger: CONTRIBUTION OF THE PARATHYROID CYP27B1 TO CIRCULATING 1,25(OH)2D LEVELS AND HYPERCALCEMIA


7. Mark Rybchyn: THE UPSTREAM AND DOWNSTREAM OF CaSR-DEPENDENT AKT SIGNALING IN HUMAN OSTEOBLASTS

8. Christian Santa Maria: SKELETAL ANABOLISM BY CONCURRENTLY TARGETING THE PTH1R AND CaSR

9. Martin Schepelmann: THE CALCIUM-SENSING RECEPTOR (CaSR) REGULATES BLOOD PRESSURE, VESSEL TONE AND CARDIAC FUNCTION

10. Polina Yarova: CALCILYTICS AS A NOVEL TREATMENT FOR ASTHMA AND COPD
ENDO Young Investigator Travel Award Winners

1. Valerie Babinsky: CASR ALLOSTERIC MODULATORS RECTIFY SIGNAL TRANSDUCTION ABNORMALITIES ASSOCIATED WITH G-PROTEIN ALPHA-11 (Gα11) MUTATIONS CAUSING FAMILIAL HYPOCALCIURIC HYPERCALCEMIA TYPE 2 (FHH2) AND AUTOSOMAL DOMINANT HYPOCALCEMIA TYPE 2 (ADH2)

2. Sarah Brennan: CALCIUM-SENSING RECEPTOR (CaSR) REGULATES HUMAN FETAL LUNG DEVELOPMENT THROUGH THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE RECEPTOR (CFTR)

3. Harry Greenberg: CALCIUM-SENSING RECEPTOR INDUCES VASORELAXATIONS VIA NITRIC OXIDE GENERATION AND INTERMEDIATE-CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS

4. Wonnam Kim: THE CALCIUM-SENSING RECEPTOR (CaSR) SUPPORTS THE GROWTH OF BREAST CANCER CELLS IN HIGH CALCIUM ENVIRONMENTS BY STIMULATING PARATHYROID HORMONE-RELATED PROTEIN (PTHrP) PRODUCTION

5. Ursula Thiem: CHANGES IN PARATHYROID HYPERPLASIA IN CINACALCET-TREATED RENAL TRANSPLANT PATIENTS: SUBGROUP ANALYSIS OF A PROSPECTIVE SELF-CONTROLLED STUDY ON THE LONG-TERM USE OF CINACALCET FOR POST-TRANSPLANT HYPERCALCEMIC HYPERPARA-ThYROIDISM
F.I.R.M.O. Young Investigator Travel Award Winners

1. Mariangela Galante: POSSIBLE IMPLICATION OF THE EGF LIGAND/EGFR SIGNALLING PATHWAY IN CASR OVEREXPRESSING BREAST CANCER CELLS OSTEOLYTIC POTENTIAL

2. Samawansha Tennakoon: CASR DEPENDENT G-PROTEIN ACTIVATION AND ITS DOWNSTREAM SIGNALLING IN THE COLON