Dietary Protein and Bone Health: Roles of Amino Acid–Sensing Receptors in the Control of Calcium Metabolism and Bone Homeostasis

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Abstract

In this article, we review the evidence that dietary protein has a positive influence on bone health, reduces hip fracture risk, and promotes post-fracture recovery, and we consider the molecular, cellular, and endocrine bases of the interactions that link protein and calcium metabolism, including effects via IGF-1 and PTH. In addition, we consider the roles of amino acid–sensing mechanisms in coupling dietary protein intake to metabolic change as well as the central role of calcium-sensing receptors (CaRs) in the control of calcium metabolism. Finally, we consider how recently identified broad-spectrum amino acid–sensing receptors from class 3 of the G-protein coupled receptor superfamily including, remarkably, the CaR itself may contribute to the impact of dietary protein on bone.

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INTRODUCTION

Synthesis of Bone Requires a Continuous Supply of Amino Acids

The net synthesis of new proteins from amino acids is a key feature of growth regardless of whether it involves the skeleton, skeletal muscle, or other tissues. In addition, proteins are inevitably turned over by proteolytic cleavage to peptides and free amino acids. In bone, the osteoclast interacts with the mineralized matrix in a cell membrane-enclosed resorption pit. First, it dissolves hydroxyapatite crystals to release Ca^{2+} and inorganic phosphate ions by acidification, a process requiring the coordinated action of a proton-pumping vacuolar adenosine triphosphatase (ATPase) (83) and a chloride (Cl^-) channel, ClCN7 (112), to provide the local production of HCl. Next, it degrades the demineralized matrix by the actions of proteases. Cathepsin K and other cysteine proteases with low pH optima appear to act first, releasing various collagen-derived peptides, including the cross-linked C-terminal peptide, CTX (for a review, see 82). Proteases with optimal activity at neutral pH, including the matrix metalloproteinases, act later to release various additional peptides. Some are bioactive (for a review, see 132); others are biologically inert and, after release into the plasma, are readily excreted by the kidneys. Fasting has an almost immediate negative impact on collagen synthesis that is independent of vitamin C status and, thus, hydroxyproline synthesis (41, 155). Similarly, low-protein diets induce reduced collagen synthesis (60); moderate- to high-protein diets, by providing high levels of amino acids, promote bone collagen synthesis (for a review, see 142).

Thus, the normal processes of bone matrix turnover result in protein degradation and amino acid losses whether by peptide excretion or hepatic deamination of free amino acids. Ingestion and digestion of dietary protein followed by the absorption of free amino acids help to redress the balance and, indeed, promote the acquisition of bone mass (for a review, see 142).

IMPACT OF DIETARY PROTEIN ON PARAMETERS OF BONE HEALTH

Given the central role of protein as a structural component of bone and new insights into its impact on growth and tissue maintenance, it is perhaps surprising that for much of the twentieth century, dietary guidelines for bone health focused on the potential harmfulness of protein intake. The basis of the difficulty is that protein, regardless of origin, is a source of metabolic acid. This is clear from the impact of dietary protein ingestion on urinary pH: Protein containing high levels of the sulfur-containing amino acids, cysteine and methionine, provides a substrate for the synthesis of sulfuric acid (for a review, see 135). In addition, dietary protein intake promotes renal calcium excretion (96, 98, 100), and the attendant hypothesis that protein-derived sulfuric acid demineralizes bone via a simple physicochemical effect was widely accepted.

Within this theoretical framework, many epidemiological studies prior to the late 1990s were undertaken with the expectation that dietary protein would be harmful for bone health and promote fracture risk. It is important to note, however, that although dietary protein intake typically lowers the pH of urine, the pH of the extracellular fluid is undisturbed due to efficient regulatory control by the kidneys (121). Furthermore, oral protein loading induces an acute rise in renal calcium excretion that peaks within 3 h (173)—too fast for an effect arising from chronic subclinical acidosis. In addition, in contrast to earlier expectations, epidemiological evidence suggests that dietary protein has a strongly positive influence on bone health (see sidebar Major Positive Effects of Protein on Bone Health).

MAJOR POSITIVE EFFECTS OF PROTEIN ON BONE HEALTH

CHILDREN AND ADOLESCENTS
Bone growth
Peak bone mass
Cortical area and strength

ADULTS
Bone mass and bone mineral density
Reduced bone loss
Recovery from hip fracture

IMPACT OF DIETARY PROTEIN ON PEAK BONE MASS AND AGE-DEPENDENT CHANGES IN BONE MASS

Age-dependent bone mass, a significant component of fracture risk, is determined by the peak bone mass achieved shortly after puberty and by the subsequent rate of bone loss (16, 17, 148). Dietary protein intake promotes peripubertal bone growth and retards bone loss (149). In addition, it appears to be necessary for optimal bone metabolism during growth and aging, favorably influencing bone mass, bone mineral density, and bone strength. It also promotes muscle mass and strength, which have an important impact on the risk of falling (for a review, see 142).

DIETARY PROTEIN AND BONE GROWTH

Protein intakes in children and adolescents influence bone growth and bone mass accumulation (15) so that variations in protein intake within the generally accepted normal range (around 0.8–1.5 g kg-body-weight^{-1} day^{-1}, but as high as 2 g kg^{-1} day^{-1} in children) have a significant impact on skeletal growth and thereby modulate the genetic potential for peak bone mass.

Prospective observational studies suggest that protein and calcium intake are independent predictors for the acquisition of bone mass (for a review, see 15). For example, spontaneous protein intake correlated positively with bone mineral density (BMD) and bone mineral content (BMC) and positively modulated the effect of calcium supplementation in prepubertal boys (38). In a prospective study of female and male subjects aged 9 to 19 years in which food intake was assessed on two separate occasions one year apart (42), a positive correlation was found between lumbar and femoral bone mass gain, on the one hand, and protein or calcium intake, on the other (15). The effects were strongest in prepubertal children, and the effect of protein intake was significant even after adjustment for calcium intake. Similar results were obtained in a prospective four-year longitudinal study of healthy children aged 6–18 in which a significant positive association was found between long-term protein intake and BMC, periosteal circumference, cortical area, and an index of strength strain (5). Overall, protein intake accounted for 3% to 4% of the variance in the measured parameters, and no association was found with the intake of sulfur-containing amino acids or calcium (5).

These results support the idea that moderate-to-high dietary protein intake promotes peak bone mass and mineralization and suggest the need for large, randomized, controlled trials to test the impact of dietary protein intake on bone mass, mineralization, and morphology.

DIETARY PROTEIN, BONE MASS, AND BONE MINERAL DENSITY

Spontaneous protein intake is positively associated with bone mass at various skeletal sites in children or adolescents (5, 26, 38), premenopausal women (49, 84, 159), postmenopausal women (36, 40, 56, 72, 86, 93, 153, 163, 167), and men (43, 174). Consistent with these reports, longitudinal follow-up data from the Framingham Study demonstrated that the rate of age-dependent bone loss was inversely correlated with dietary protein intake (77). In another longitudinal study, spontaneous protein intake was positively associated with change in BMD in patients receiving calcium supplements but not placebo, which

suggests that dietary protein positively modulates calcium absorption and/or metabolism (50, 51).

In contrast, very few studies have concluded that protein intake is negatively associated with bone mass. Data from the Study of Osteoporotic Fractures suggests that unadjusted BMD was greater in the group with higher protein intakes, but the research group concluded that a high ratio of animal to plant protein may be harmful for BMD and hip fracture risk (153). In another cross-sectional study, a protein intake close to 2 g kg^{-1} day^{-1}, which is often considered high in adults, was associated with reduced bone mineral density at one of two forearm sites in healthy young women (124).

Taken together, the results strongly support a positive impact of dietary protein on bone mass and BMD and indicate that although a decline in calorie intake may be appropriate with advancing age, a parallel reduction in protein intake is detrimental to bone health. In addition, the results indicate that the positive effects of protein depend, at least in part, upon an adequate intake of calcium.

DIETARY PROTEIN AND FRACTURE RISK

It has been noted that, in general, countries with the highest intakes of animal protein have the highest hip fracture rates (2, 67) and the longest life expectancies—suggesting that, at the population level, protein intake and fracture risk are spuriously linked via age. In the Nurses' Health Study, hip fracture incidence trended inversely with protein intake (63). The same study, however, reported an increased forearm fracture risk in subjects with the highest intake of animal protein in whom fruit and vegetables, which are important sources of vitamins and other micronutrients, may have been displaced from the diet. In contrast, in a prospective study of approximately 40,000 women in Iowa, higher protein intakes were associated with a reduced risk of hip fracture (127), and the protective effect was observed with protein of animal origin. Similarly, a case-control study of 2500 men and women in Utah demonstrated a negative relationship between protein intake and hip fracture incidence in men and women between the ages of 50 and 69. In the highest quartile of protein intake, hip fracture incidence was reduced by an impressive 65% (172). In another study, no association between hip fracture and nondairy animal protein intake was detected (125); however, fracture risk increased when high dietary protein intake was accompanied by low calcium intake, supporting the notion that the positive effects of dietary protein are dependent on calcium. A synergistic interaction between protein and calcium might operate most obviously at the cellular level, e.g., at or near the small intestinal site of calcium absorption and/or at the site of osteoblast-dependent bone matrix synthesis and mineralization. As described below, it is now known that positive interactions between protein/amino acids and calcium can also occur at the molecular level.

Effects of Correcting Protein Insufficiency on Postfracture Recovery

Intervention studies using oral protein preparations report significantly improved clinical outcomes after hip fracture (55, 152, 164). In these studies, a dietary protein supplement of 20 g was selected to increase protein intake to a level at, or just below, the recommended daily allowance for sedentary adults (0.8 g kg-body-$weight^{-1}$ day^{-1}; for a review, see 29), thereby avoiding the risk of dietary protein overload, which is potentially harmful in patients with hepatic and/or renal impairment. At follow-up, the supplemented patients had lower rates of complications including bedsores, anemia, and intercurrent lung or renal infections, and they had lower death rates (164). Furthermore, durations of stay in both the orthopedic ward and convalescent hospital were significantly reduced. In a subsequent six-month, double-blind, isocaloric placebo-controlled trial in elderly patients who all received 200,000 international units vitamin D by intramuscular injection at baseline and 550 mg day^{-1} of calcium, a daily protein supplement of 20 g produced gains in serum

insulin-like growth factor-1 (IGF-1), prealbumin, and immunoglobulin M levels, and attenuated a time-dependent decrease in proximal femur BMD (152).

ORGANIZATION OF MINERAL METABOLISM

The coordinated activities of various cell types in diverse tissues, including the gastrointestinal tract, kidneys, skeleton, and regulatory organs and tissues, are required for the normal control of mineral metabolism and bone homeostasis. This activity (*a*) permits a mechanically competent expansion of bone tissue during growth; (*b*) supports selective repair of bone tissue in response to focal damage arising from wear and tear; and (*c*) is tightly linked to homeostatic mechanisms for extracellular Ca^{2+} ($Ca^{2+}{}_o$) and inorganic phosphate whereby, regardless of whether the skeleton is at steady-state or undergoing net accumulation or loss of mineral, the concentrations of ionized Ca^{2+} and inorganic phosphate are restricted to tightly defined normal ranges: 1.1–1.3 mM for $Ca^{2+}{}_o$ and 0.8–1.4 mM for phosphate.

Normal mineral metabolism requires regulated:

- release and solubilization of calcium and phosphate from ingested foods, facilitated by gastric acid and digestive enzymes;
- intestinal absorption of calcium and phosphate;
- renal excretion of calcium and phosphate; and
- formation and turnover of calcium- and phosphate-containing crystals in the bone matrix.

In functional terms, tissues that participate in whole-body calcium and/or phosphate metabolism (*a*) coordinate hormonal responses (parathyroid, thyroid C-cells, renal proximal tubular cells, and possibly osteocytes in bone) (61, 119), (*b*) transport Ca^{2+} and inorganic phosphate as well as other nutrients, water, and electrolytes into and/or out of the body (small intestine and kidney), (*c*) respond to Ca^{2+} ions and other nutrients with modified rates of growth, differentiation or turnover (bone cells and their precursors as well as parathyroid cells), or (*d*) utilize Ca^{2+} and phosphate as key structural components (bone). Many of these functions are regulated or modulated by calcium- or phosphate-sensing mechanisms, e.g., the extracellular Ca^{2+}-sensing receptor.

After ingestion, poorly soluble salts of calcium and phosphate (e.g., Ca_2HPO_4 or Ca_3PO_4) have multiple, alternative fates. They might:

- be rendered, or remain, insoluble (e.g., due to inadequate gastric acid production) and thus excreted in the feces;
- be solubilized and either absorbed in the small intestine (if the capacity of the uptake pathway is adequate) or excreted in the feces;
- if absorbed, be distributed in the blood to the kidneys for recycling or excretion; be sequestered in intracellular stores; or be precipitated after uptake into bone matrix vesicles or incorporated extracellularly into expanding hydroxyapatite crystals on preformed nuclei.

How are these processes regulated, on the one hand, to provide homeostatic control over systemic $Ca^{2+}{}_o$ and phosphate concentrations and, on the other, to couple nutritionally derived signals to promote bone growth in children and maintain bone mass and integrity in adults? In particular, what determines the relative distribution of calcium and phosphate ions between these various fates? The hormonal regulation of mineral metabolism by parathyroid hormone (PTH) (targeting kidney and bone), calcitonin (targeting bone), and calcitriol (targeting the gut) is well known. Brief descriptions of the major effects of these hormones follow.

Calciotropic Peptide Hormones: PTH and Calcitonin

PTH differentially regulates renal Ca^{2+} and phosphate reabsorption, acting to promote Ca^{2+} reabsorption and suppress phosphate reabsorption, thereby inducing phosphaturia.

When chronically elevated, PTH also promotes proximal tubular synthesis of calcitriol and elicits osteoblast-dependent recruitment and activation of osteoclasts to drive bone resorption—although in pharmacological doses, repetitive acute exposure of osteoblasts to PTH promotes accumulation of bone mass and bone mineralization (87). Calcitonin is derived from thyroid parafollicular C-cells. It exerts a negative influence on osteoclasts to suppress bone resorption.

Sterol Hormone: Calcitriol

The hormonally active form of vitamin D, calcitriol (1,25 dihydroxyvitamin D), acts primarily on the small intestine to promote absorption of calcium and phosphate from ingested foods. Ca^{2+} entry from the luminal fluid into the cytoplasm of small intestinal epithelial cells occurs via the Ca^{2+}-permeable channel, TRPV6 (89). Ca^{2+} then traffics to the basolateral (blood-facing) region of the cell bound to a molecular chaperone, calbindin D_{9K}, and is extruded by a plasma membrane Ca^{2+}-ATPase (PMCA) and/or Na^{+}-Ca^{2+} exchanger (161). Calcitriol also activates a transport pathway for inorganic phosphate (for a review, see 139). Together, the absorption of calcium and phosphate is critical for bone growth and maintenance. However, as discussed below, it seems clear that the serum calcitriol level is not the sole determinant of calcium and phosphate absorption. Additional hormonal influences on bone and mineral metabolism are also recognized, including sex steroids such as estrogen and testosterone as well as growth factors such as IGF-1 (gut and bone; see below) and some fibroblast growth factors, notably FGF-23 (139).

THE IMPACT OF DIETARY PROTEIN INTAKE ON CALCIUM METABOLISM

Molecular Signals Derived from Dietary Proteins

How does dietary protein modulate bone and mineral metabolism to influence bone health? Proteins are diverse in their amino acid sequences and their tertiary structures. It seems doubtful, therefore, that proteins per se have any impact on whole-body macronutrient or mineral metabolism. After ingestion, however, proteins are digested to release short peptides and free amino acids and then absorbed by small intestinal epithelial cells. In general, peptides are not released into the blood but rather are broken down intracellularly by the action of peptidases to free amino acids for transfer into the blood (3, 4). Thus, within the gut lumen, protein-derived chemical signals can take the form of peptides (e.g., phosphopeptides derived from casein; 151) and free amino acids, but in the blood, protein-derived chemical signals are likely to take the form of free amino acids or their metabolites.

Impact of Protein and Amino Acids on Calcium Absorption and Excretion

Although amino acids and calcium belong to distinct nutrient classes, both are key structural elements of the skeleton and both are found enriched in important growth-promoting foods, e.g., milk and meat. It is perhaps not entirely surprising, therefore, that variations in the level of dietary protein ingestion have a significant impact on whole-body calcium metabolism or that the positive effects of dietary protein intake on bone health appear to be dependent, at least in part, on calcium intake (50, 51, 125). The acute effects of increased dietary protein intake in both humans and other mammals include enhanced intestinal calcium absorption (102, 103) (**Figure 1**) as well as enhanced renal calcium excretion (6, 98, 100). However, enhanced calcium absorption was not observed in a longitudinal study of nearly 200 women aged 35–45 at the time of recruitment (79).

Impact of Dietary Protein on Serum Parathyroid Hormone Levels

On the other hand, careful studies demonstrate that reduced dietary protein intake in

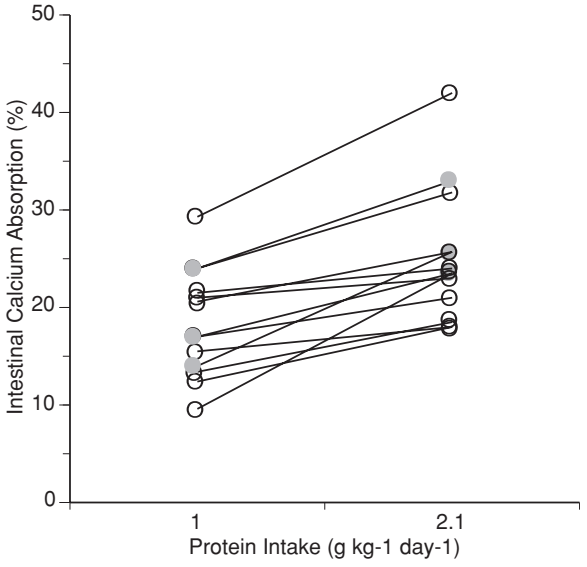

Figure 1

Impact of an increase in dietary protein intake on intestinal calcium absorption. Ten healthy premenopausal women (ages 20–40; open circles) and three healthy postmenopausal women (ages 55–70; shaded circles) were studied in two separate cycles (102). In each cycle, dietary protein intake was first adjusted to around 1.2 g kg^{-1} day^{-1} for two weeks and then tightly controlled during the following 10-day experimental period at either 1.0 g kg^{-1} day^{-1} or 2.1 g kg^{-1} day^{-1} by the provision of all foods from a metabolic kitchen. Protein was derived from both plant and animal sources, and total calorie intake was constant. Dietary supplements of vitamins or minerals were suspended for the course of the study. %Absorption was calculated using a dual stable isotope technique based on the relative recovery of the oral to intravenous calcium tracer in 34-h urine collections obtained postdosing (102). Redrawn with the permission of the authors and *Journal of Clinical Endocrinology and Metabolism*, copyright 2005, the Endocrine Society.

humans promptly induces a state of secondary hyperparathyroidism over 2–3 days that is typified by normal serum total and ionized Ca^{2+} concentrations and elevated serum PTH levels (101, 104) in healthy young subjects consuming dietary protein at a level below 0.9 g kg^{-1} day^{-1} (104). Secondary hyperparathyroidism with normal serum Ca^{2+} levels is commonly ignored in clinical practice provided renal function is normal and vitamin D status is satisfactory (serum 25-hydroxyvitamin D $\geq$60 $nmolL^{-1}$) but may be harmful due to prolonged skeletal exposure to elevated PTH levels. Consistent with this more disturbing view, low dietary protein-induced elevations in serum PTH levels were associated with secondary elevations in serum calcitriol and urinary cyclic AMP levels, demonstrating that type-1 PTH/PTHrP receptors had been activated in the kidney and, presumably, other tissues including osteoblasts in bone (101). Although the molecular and cellular bases of these effects are not known, these findings indicate that protein and calcium metabolism are coordinated and that calcium metabolism is modulated by variations in protein intake (**Table 1**).

THE IMPACT OF DIETARY PROTEIN ON HORMONAL REGULATORS OF MACRONUTRIENT METABOLISM

Dietary protein or amino acid intake promotes the release of insulin (28, 64, 65) as well as the key growth factor IGF-1 (95). In addition, ingestion of dietary protein or administration of amino acids, including the basic amino acid arginine, stimulates growth hormone (GH) secretion (109), which promotes IGF-1 production from the liver and other tissues. Consistent with these effects, dietary protein restriction reduces plasma IGF-1 levels as a result, at least in part, of hepatic resistance to GH action and enhanced metabolic clearance (for reviews, see 162, 168).

Significance of IGF-1 for Bone Growth and Maintenance

IGF-1 is an essential factor for longitudinal bone growth (reviewed in 69) and exerts anabolic effects on bone mass during adulthood (reviewed in 54). It has pluripotent effects on calcium and phosphate metabolism, including enhanced calcitriol synthesis and stimulated renal phosphate reabsorption (30, 133). IGF-1 also selectively stimulates the plasma membrane uptake of inorganic phosphate in osteoblastic cell lines (134), which promotes mineralization, and based on analyses in knockout mice, both IGF-1 and expression of IGF-1

Table 1 Impact of dietary protein on calcium metabolism

Tissue	Impact of protein or amino acids	Significance for calcium metabolism	Putative amino acid sensor(s)	References
Modulation of hormonal control				
Pituitary	Enhanced growth hormone secretion	Increased serum IGF-1	Basic amino acid sensor (? GPRC6A)	109
Parathyroid	Suppressed PTH secretion	Reduced osteoblast-dependent bone turnover	CaR	47, 101, 104
Modulation of calcium absorption and excretion				
Stomach	Enhanced acid secretion	Increased calcium solubility	CaR; L-type amino acid transporter	27, 107
Duodenum	Enhanced calcium absorption	Increased calcium availability	CaR in stomach (? also small intestine)	102, 103
Renal tubules	Enhanced calcium excretion	? minimal (secondary to enhanced calcium absorption)		6, 98, 100
Modulation of the target organ				
Bone	Enhanced local IGF-1 production; enhanced osteoblast maturation and function	Enhanced osteoblast cell number; bone matrix synthesis and mineralization	Basic amino acid sensor (? GPRC6A) ? CaR	39

Question marks indicate uncertainty. GPRC6A, G protein–coupled receptor, family C, group 6, member A; IGF-1, insulin-like growth factor-1; PTH, parathyroid hormone.

receptors on osteoblasts are required for the anabolic effect of acutely administered PTH (12, 170). In addition to its systemic production in the liver under GH control, IGF-1 is produced by osteoblastic cells in response to free amino acids including arginine (39), and in recently completed, short-term studies on elderly subjects, a protein supplement of 20 g day^{-1} significantly increased serum IGF-1 and IGF-binding protein-3 levels within a week (R. Rizzoli, unpublished findings).

To further investigate the interactions between protein nutrition, IGF-1 status, and bone homeostasis, Rizzoli and colleagues developed an experimental model of selective protein deprivation in adult female rats (8, 18). The milk protein casein was used as the primary protein source, and the following parameters were examined: bone mass, bone mineral density, bone strength, and bone remodeling. An isocaloric, low-protein diet induced a decrease in BMD at skeletal sites formed by either trabecular or cortical bone, associated with a marked and early decrease in serum IGF-1 levels that fell by approximately 40% over 14 days (18). Subsequent administration of essential amino acid supplements in the same relative proportion as casein caused a prompt increase in the serum IGF-1 level along with increased markers of bone formation and decreased markers of bone resorption (7, 9). Interestingly, bone strength and cortical thickness increased markedly (9). In other experiments, adult male rats developed osteoporosis on low-protein diets in association with reductions in serum IGF-1 levels (19). Based on these observations in rats as well as the human studies referred to above, IGF-1 appears to play a prominent role in maintaining normal bone health, and reductions in IGF-1 levels appear to increase the risk of osteoporosis and associated fractures. In addition, IGF-1 levels respond sensitively and positively to changes in dietary protein and amino acid intake.

Thus, it is evident that dietary protein intake and protein-derived amino acids modulate calcium metabolism and bone homeostasis via effects on calcium absorption and excretion as well as the hormonal and growth factor milieu.

In the subsequent sections, we consider how amino acid–sensing mechanisms contribute to the control of cellular metabolism.

ROLES OF AMINO ACID SENSORS IN COUPLING DIETARY PROTEIN INTAKE TO METABOLIC CHANGE

Variations in the serum or intracellular concentrations of free amino acids provide signals leading to changes in the levels of hormones that modulate digestion, absorption, satiety and appetite, nutrient disposal, metabolic rate, and fuel selection. Identifying amino acids as signals in this way is analogous to the role of glucose in signaling the state of whole-body carbohydrate stores. Following a carbohydrate-rich meal, the plasma glucose level normally rises by 1.5- to 2-fold, from approximately 3–4 mM to 6–7 mM. Similarly, following a protein-rich meal, free amino acid levels in systemic plasma rise by approximately 1.5-fold, depending upon the protein source and its amino acid composition (20, 62, 123). However, much larger increases in serum amino acid concentrations of 2- to 3-fold have been observed in humans following the ingestion of peptide hydrolysates or free amino acids (28, 75). Although glucose acts as the key signal of carbohydrate ingestion, protein ingestion is reported by as many as 20 distinct amino acids. As a result, amino acid–sensing mechanisms are often more promiscuous, recognizing one or more subclasses of amino acids rather than individual free amino acids.

CELLULAR AMINO ACID–SENSING MECHANISMS

The detection of changes in amino acid levels requires cellular amino acid–sensing mechanisms that, until recently, have been poorly defined. Analyses of the mechanisms that determine food selection and foraging behavior in the central nervous system of rodents (reviewed in 73), protein synthesis in muscle (reviewed in 106), and suppression of hepatic autophagy (reviewed in 169) have provided important insights. In the case of the mammalian piriform cortex, which controls feeding behavior, an intracellular amino acid–sensing mechanism operates in which cytoplasmic levels of amino acid–free tRNAs drive the activation of an intracellular protein kinase, GCN2 (73). Remarkably, it closely resembles an intracellular amino acid–sensing mechanism used by yeast to control amino acid biosynthesis (128). In the case of mammalian muscle protein synthesis, nutrient-dependent signals including free amino acids as well as insulin and IGF-1 control the activity of an intracellular protein complex, mammalian target of rapamycin complex 1 (mTORC1), that contains the serine/threonine kinase mTOR and regulates translation via the protein kinase S6 (106). Intriguingly, full activation of the complex requires at least two different amino acids (e.g., glutamine and leucine) and/or closely related metabolites, implying the existence of a strong positive interaction between two distinct nutrient sensors (106).

Intracellular and Extracellular Amino Acid Sensing

Amino acid sensors are located either intracellularly or extracellularly. As noted above, one form of intracellular amino acid–sensing mechanism is based on amino acyl–free tRNAs (for reviews, see 73, 78). Extracellular amino acid–sensing mechanisms, on the other hand, appear to be based either on surface membrane receptors (reviewed in 45) or transporters (reviewed in 92) and provide information on extracellular levels of free amino acids. Receptors couple to cellular responses by intracellular signaling pathways, and a group of broad-spectrum amino acid–sensing G-protein-coupled receptors has recently been identified (45). Transporters, in addition to their more obvious roles in facilitating transmembrane fluxes, can also couple to intracellular signaling pathways either directly or indirectly, e.g., secondary to the activation of amino acid–sensing receptors following cellular export.

A possible link between amino acid transport and osteoblast-dependent collagen synthesis

was recently identified in mice homozygous for ablation of the transcription factor ATF4; amino acid transporter expression is defective in these mice, which exhibit delayed skeletal development and high levels of fetal wastage. Interestingly, high-protein diets normalized the phenotype and promoted survival (60). In the yeast *Saccharomyces cerevisiae*, one well-characterized system for the detection of extracellular amino acids is based on a plasma membrane complex of three proteins, Ssy1p, Ptr3p, and Ssy5p, which controls the expression of genes required for amino acid synthesis, metabolism, and transport (57, 66, 108). Intriguingly, Ssy1p is a member of a family of amino acid permeases that appears to have lost its transport function in the process of developing a role in amino acid sensing. In two mammalian examples, glutamate transport appeared to induce the activation of ERK1/2 in rat astrocytes (1), and an amino acid transport inhibitor 2-amino, bicyclo (2,2,1) heptane-2 carboxylic acid blocked the activation of acid secretion from rat parietal cells by some (107), but not all (27), amino acids.

Thus, amino acid sensing can be mediated, at least in part, by amino acid transporters; however, the underlying mechanisms and the nature of the secondary roles of receptors are unclear. In contrast, the signaling mechanisms that underlie amino acid sensing by membrane receptors together with their physiological significance are better defined. For this reason, the subsequent sections focus on the nature and roles of class 3 G-protein coupled receptors (GPCRs), which include a subgroup of broad-spectrum amino acid sensors.

BROAD-SPECTRUM AMINO ACID SENSING BY CLASS 3 GPCRS

Class 3 (family C) GPCRs are encoded by approximately 20 genes (see review in 21). Like other GPCRs, they are typified by a seven transmembrane domain-signaling motif for the binding and activation of heterotrimeric G-proteins but are unusual in exhibiting a markedly extended N-terminus of about 400–600 amino acids (13). The extreme N-terminus is recognized as a distinct structural domain of approximately 400–500 amino acids that is bilobed and related to bacterial periplasmic-binding proteins that mediate biochemical interactions between nutrients and transmembrane proteins involved in transport and/or intracellular signaling (131). This domain is now widely referred to as the Venus Flytrap (VFT) domain in recognition of its bilobed, nutrient-trapping structure. Class 3 GPCRs are thus eukaryotic descendants of an ancient nutrient-sensing system with diverse applications in human biology.

The first recognized members of GPCR class 3, the metabotropic glutamate receptors and γ-aminobutyrate (GABA) B receptors, are not broad-spectrum amino acid sensors and do not appear to have a defined role in nutrient sensing. Nevertheless, glutamate is an acidic amino acid and GABA is a glutamate analog derived by the action of glutamate decarboxylase on the amino acid's α-carboxylate group. Recent work demonstrates that members of one subgroup of GPCR class 3, however, are much more promiscuous, sensing and responding to several subgroups of amino acids (for a review, see 45). They include a heterodimeric taste receptor, composed of T1R1 and T1R3 receptor subunits, which responds to aliphatic, polar, charged, and branched-chain amino acids (129); the goldfish 5.24 receptor and its mammalian ortholog, G protein-coupled receptor, family C, group 6, member A (GPRC6A), which respond to basic amino acids such as arginine and lysine as well as aliphatic and polar amino acids (115, 156, 171); and, remarkably, the extracellular Ca^{2+}-sensing receptor (CaR) that, in addition to its agonist binding sites for $Ca^{2+}{}_o$, exhibits an allosteric site for aromatic, aliphatic, and polar amino acids in its VFT domain (48; **Figure 2**).

The CaR is expressed in tissues and cell types with recognized roles in amino acid sensing as well as calcium sensing, including enzyme and hormone-secreting cells of the stomach and small intestine, the liver, exocrine and endocrine cells of the pancreas, and endocrine

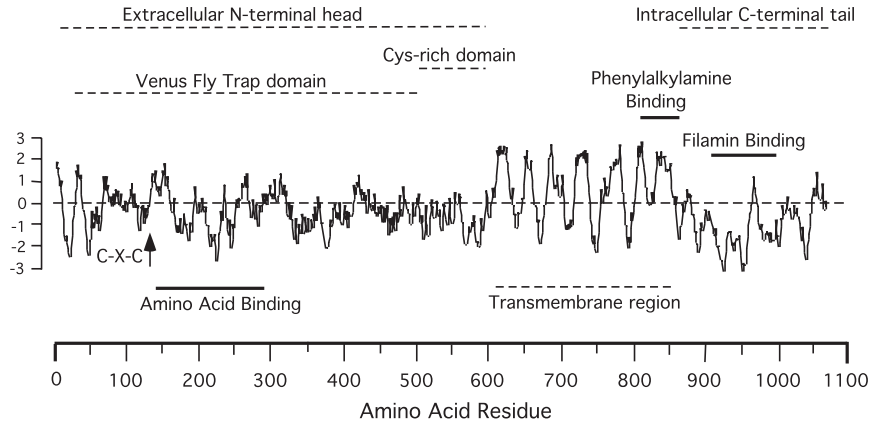

Figure 2

Annotated map of the calcium-sensing receptor. An annotated hydropathy plot (Kyte-Doolittle) of the human Ca^{2+}-sensing receptor is used to show the positions of the N-terminal Venus Flytrap domain, Cys-rich domain, seven-transmembrane domain region, and C-terminal signaling and cytoskeletal-coupling domain. The recognized binding sites for amino acids in the Venus Flytrap domain and phenylalkylamine type-II calcimimetics such as cinacalcet are also shown (from 44). C-X-C: location of disulfide linkages in receptor homodimers.

cells of the anterior pituitary (for a review, see 90). By binding and responding to Ca^{2+} ions in an amino acid–dependent manner, the CaR appears to provide a direct molecular link between protein and calcium metabolism.

REGULATORY AND MODULATORY ROLES OF THE EXTRACELLULAR Ca^{2+}-SENSING RECEPTOR IN MINERAL METABOLISM AS WELL AS SKELETAL DEVELOPMENT, GROWTH, MAINTENANCE, AND TURNOVER

CaR-expressing tissues operate in two distinct modes, either by (*a*) sensing and normalizing inappropriate fluctuations in Ca^{2+}_o and adjusting the serum inorganic phosphate level or by (*b*) supporting the development, growth, maintenance, and/or turnover of the skeleton (some renal tubular cells, the gastrointestinal tract, thyroid C-cells, and possibly bone and cartilage). In this latter mode, the size of the skeletal calcium store in the form of matrix-associated hydroxyapatite can be adjusted either up or down, thereby influencing bone mass, bone quality, and bone health. Under conditions of chronic stress, the distinction between these two modes of operation breaks down because the skeleton, as the ultimate bodily calcium and phosphate store, can be recruited to the support of extracellular Ca^{2+}_o and inorganic phosphate homeostasis, e.g., in the context of persistent hypocalcemia and secondary hyperparathyroidism.

Tissues That Sense Changes in Ca^{2+} and Coordinate Homeostatic Responses

Parathyroid and thyroid C-cells. The chief cells of the parathyroid glands express the CaR at robust levels (105). The CaR mediates high Ca^{2+}_o-evoked suppression of PTH secretion, down-regulates PTH gene expression (117), and inhibits parathyroid cellular proliferation in humans (160) and mice (85). Reduced CaR expression or function arising from mutations of the CaR gene accounts for around 90% of cases of familial hypocalciuric hypercalcemia cases in humans (160). In addition, humans and mice homozygous for inactivating mutations of the CaR manifest severe primary

hyperparathyroidism due to extreme resistance to the inhibitory action of high Ca^{2+}_o on PTH release (85, 160). In response to the marked elevation of serum PTH levels, there is inappropriately high renal tubular Ca^{2+} reabsorption, phosphate wasting, and bone demineralization and resorption.

Contrary to its inhibitory effect on PTH release, elevated Ca^{2+}_o stimulates calcitonin (CT) secretion (reviewed in 24), which in turn acts to suppress Ca^{2+}_o, primarily via an antiresorptive action on osteoclasts, which express CT receptors (reviewed in 91). Thyroid C-cells have been shown to express the CaR (24, 68), and CaR cDNAs cloned from rat kidney and a rat C-cell line were identical (71, 146). Furthermore, an analysis of heterozygous CaR-null mice indicates that the receptor normally mediates high Ca^{2+}_o-stimulated secretion of CT (70).

Renal tubules. In the rat kidney, the CaR is expressed throughout the nephron, with highest expression on the basolateral surfaces of cortical thick ascending limb (CTAL) and distal convoluted tubule cells (145), which support PTH-regulated reabsorption of divalent cations (53) and respond to hypercalcemia with suppressed Ca^{2+} reabsorption. The CaR is also expressed in the proximal tubule, where it attenuates the phosphaturic effect of PTH (10) and lowers serum calcitriol levels at least in part by increasing vitamin D receptor expression (122). Interestingly, CaR expression in the proximal tubule is under the inhibitory control of dietary phosphate and PTH (147).

Tissues That Support Skeletal Development, Growth, Maintenance, and Mineralization

Stomach and small intestine. Reduced bone mineral density is a known long-term side effect of total or partial gastrectomy (14, 81, 140), and proton pump inhibitors interfere with the absorption of Ca^{2+} ions from some calcium salts (130). Furthermore, a recent study indicates that there is a substantial drop in the solubility of various calcium salts above pH 6.0 (74), the approximate pH of the duodenum following the entry of gastric acid. Since calcium and phosphate absorption occurs primarily in the duodenum, these findings indicate that gastric acid production plays a significant role in the release and solubilization of Ca^{2+} ions from ingested food. Gastric acid production is stimulated not only by the activity of the parasympathetic nervous system but also by chemical signals including gastrin and its local effector histamine, nutrients including Ca^{2+}_o (76), and some amino acids (94, 110, 111, 157). The effects of Ca^{2+}_o are mediated, at least in part, by the CaR, which is expressed on gastric antral G-cells (141), thereby controlling the release of gastrin (25), and parietal cells (37, 58), providing a mechanism by which Ca^{2+}-rich foods can directly promote acid secretion. Duodenal calcium and phosphate absorption is also promoted by calcitriol, which upregulates proteins constituting a transcellular pathway of calcium absorption (23). Calcitriol synthesis is negatively regulated by the CaR in the context of hypercalcemia both directly via effects on proximal tubular epithelial cells and indirectly via inhibition of PTH release.

The CaR is expressed along the entire rat intestine (35) primarily on the basal surfaces of the small intestinal villous epithelial cells as well as secretory cells of the small and large intestinal crypts, some enteroendocrine cells, and in the enteric nervous system (35). It is not yet clear whether intestinal CaRs contribute to the control of systemic calcium metabolism; however, hypocalcemia and hypercalcemia increase and decrease, respectively, intestinal motility (23), and hypercalcemia decreases absorption of dietary calcium (114). Intriguingly, recent work on CYP27B1 (1α-hydroxylase)-null mice, which cannot synthesize calcitriol, demonstrates that dietary calcium supplements normalize impaired expression of TRPV6, calbindin D_{28K}, the Na^+-Ca^{2+} exchanger NCX1, and the PM Ca^{2+}-ATPase PMCA1b, together with the serum calcium concentration (88, 161). These findings suggest that the gastrointestinal tract directly senses and responds to changes in luminal Ca^{2+}_o concentration.

Bone and Cartilage: Impact of Variations in Ca^{2+}_o

The level of Ca^{2+}_o within the bone microenvironment appears to fluctuate considerably during osteoclastic bone resorption and subsequent osteoblast-dependent bone formation (for a review, see 24). Beneath resorbing osteoclasts, the Ca^{2+}_o concentration can reach 8–40 mM (154). Elevated Ca^{2+}_o modulates several functions of osteoblasts, their precursors, and osteoblastic cells in vitro that may be of physiological relevance, including enhanced proliferation and chemotaxis, augmented maturation, and enhanced mineralization (59, 137, 158, 175). In addition, elevated Ca^{2+}_o suppresses both the formation and the activity of osteoclasts in vitro (99, 120, 177; for a review, see 178). If these effects also occur in vivo, local elevations in Ca^{2+}_o could contribute to the mechanism by which bone resorption is coupled to local bone repair. However, Ca^{2+}_o activates the CaR and has significant effects on bone cell function at concentrations even in the normal physiological range (59). These findings suggest that the CaR also exerts important modulatory effects that are independent of local changes in Ca^{2+}_o concentration. Under these conditions, CaR activity might respond to variations in the levels of other activators, e.g., amino acids.

The Roles of CaRs and Other Putative Extracellular Ca^{2+} Sensors in Bone and Cartilage

The status of the CaR in the physiological regulation of bone cells has been uncertain. Some investigators have not detected CaR expression in osteoblast-like or osteoclast-like cells, suggesting instead the existence of a distinct Ca^{2+}_o-sensing mechanism. One potential candidate is the basic amino acid–sensing class 3 GPCR, GPRC6A, a close relative of the CaR, which is expressed by osteoblasts and exhibits Ca^{2+}_o-sensing properties. Other studies, however, have reported that the CaR is expressed in various cell types from bone or bone marrow, including cells of the osteoclast and osteoblast lineages (33, 59). Establishing the functional significance of CaR expression in bone cells has been complicated. The global exon-5 CaR null mouse exhibits severe primary hyperparathyroidism (85). In contrast, two double-knockout mice, exon-5 CaR/Gcm2 (166) and exon-5 CaR/PTH (113), which do not exhibit primary hyperparathyroidism due to the loss of PTH, do not exhibit a major bone phenotype. More recent work, however, indicates that exon-5 may not be required for CaR function in cells of the osteoblast lineage (150); conditional ablation of CaR exon-7, which encodes the entire 7-transmembrane domain region and carboxy-terminus, in cells that express the type-I collagen promoter (e.g., immature osteoblasts) results in a distinct murine phenotype that takes the form of growth retardation and skeletal demineralization without hyperparathyroidism (32).

Chondrocytes participate in skeletal development and longitudinal growth of bones. Ca^{2+}_o is an essential nutrient for normal growth and differentiation of chondrocytes and skeletal growth in vivo (97), and hypertrophic chondrocytes of the growth plate express the CaR (33). In studies of RCJ3.1C5.18 chondrocytes, elevated Ca^{2+}_o suppressed the expression of early markers of differentiation, including aggrecan and alkaline phosphatase (31), and promoted mineralization as well as the expression of late markers of differentiation, including the bone matrix proteins osteopontin, osteonectin, and osteocalcin (34). All these effects appeared to be CaR dependent (31, 34), and a recent abstract reports that conditional ablation of CaR exon-7 in murine chondrocytes is lethal during embryonic development (165).

AMINO ACID SENSING BY THE CALCIUM-SENSING RECEPTOR

The CaR senses amino acids in addition to Ca^{2+}, Mg^{2+}, and other multivalent cations. Comparisons between L- and D-amino acids clearly demonstrate that the CaR is stereoselective for natural (L-) amino acids and demonstrate the existence of a specific binding site. Although it is not a universal amino acid sensor,

analysis in CaR-expressing HEK293 cells (48) and human parathyroid cells (47) indicates that the CaR responds sensitively to about one-third of the 20 common amino acids and less sensitively to another one third. Based on cellular assays, the most potent amino acids are the aromatics, L-Phe, L-Trp, L-Tyr, and L-His, as well as the aliphatic and polar amino acids, L-Thr, L-Ser, and L-Ala (47, 48). The least-potent amino acids are the branch-chain subgroup, including L-Leu, L-Ile, and L-Val, the basic amino acids L-Arg and L-Lys, and the long sulfur-containing, hydrophobic amino acid L-Met. However, these differences in potency need to be qualified, first by recognizing that serum levels are generally much lower for aromatic than aliphatic or polar amino acids (compare fasting L-Phe and L-Trp levels, which are approximately 50 $\mu molL^{-1}$, with fasting L-Ala and L-Thr levels, which are approximately 300 $\mu molL^{-1}$) and second by the recognition that additional amino acids become effective as the extracellular Ca^{2+} concentration rises above 1.0 mM (47; reviewed in 45). Amino acids markedly enhance intracellular Ca^{2+} mobilization that primarily takes the form of enhanced $Ca^{2+}{}_o$ sensitivity in CaR-expressing HEK293 cells (48) as well as enhanced $Ca^{2+}{}_o$ sensitivity and increased efficacy in human parathyroid cells (47). Amino acids also induce more subtle effects on other intracellular signaling enzymes such as ERK1/2 (116). In this way, the pharmacological behavior of the amino acid–activated CaR is distinct from the $Ca^{2+}{}_o$-activated CaR, which activates multiple intracellular-signaling pathways with apparently comparable efficacy (116, 144, 176). Furthermore, although the CaR is activated by $Ca^{2+}{}_o$ in the absence of amino acids, it is only activated by amino acids in the presence of $Ca^{2+}{}_o$ concentrations above a threshold level of about 0.5–1.0 mM in CaR-expressing HEK293 cells and human parathyroid cells (for reviews, see 44, 45). This indicates a distinction in the pharmacological mechanism of action: Multivalent cations such as $Ca^{2+}{}_o$ are agonists; L-amino acids are allosteric activators that enable distinct signaling mechanism(s). Analysis of the L-amino acid–activated signaling mechanism in CaR-expressing HEK293 cells indicates that it recruits distinct protein partners including elements of the cytoskeleton and, possibly, a specific subset of Ca^{2+} channels and transporters that supports a distinctive low-frequency pattern of oscillations in cytoplasmic-free Ca^{2+} concentration (143, 144; reviewed in 22).

IMPACT OF AMINO ACID–ACTIVATED CaRs ON CALCIUM METABOLISM

As described above, the CaR is expressed in various tissues that contribute to the control of whole-body calcium metabolism. Its effects can be considered at various levels: the gastrointestinal tract, including the stomach and small intestine, in which CaRs appear to modulate calcium absorption; calciotropic hormone-secreting cells, including parathyroid cells (PTH), thyroid C-cells (calcitonin), and renal proximal tubular cells (calcitriol), in which activated CaRs adjust the balance in favor of calcitonin and away from PTH and calcitriol; cells of the CTAL and distal tubule, which control calcium reabsorption; cells of the proximal tubule that control phosphate reabsorption; and osteoblasts and osteoclasts, which control bone mass, mineralization, turnover, and repair.

Which of these effects are sensitive to the amino acid–activated as well as—or instead of—the $Ca^{2+}{}_o$-activated CaR and thus may provide an alternative mode of CaR-dependent regulation? Does the amino acid–activated CaR provide a mechanism by which calcium ions can be directed to bone at the same time that the Ca^{2+}-activated CaR protects calcium homeostasis?

CaRs promote gastric acid secretion directly via expression on the basolateral membrane of parietal cells from where they activate the proton-pumping H^+/K^+-ATPase of the apical membrane and indirectly via the release of gastrin from G-cells in the gastric antrum (for a review, see 44). Both these responses are dependent upon intracellular Ca^{2+} signaling, which as noted above is powerfully stimulated by L-amino acid–activated CaRs. In addition, aromatic amino acids such as L-Phe and

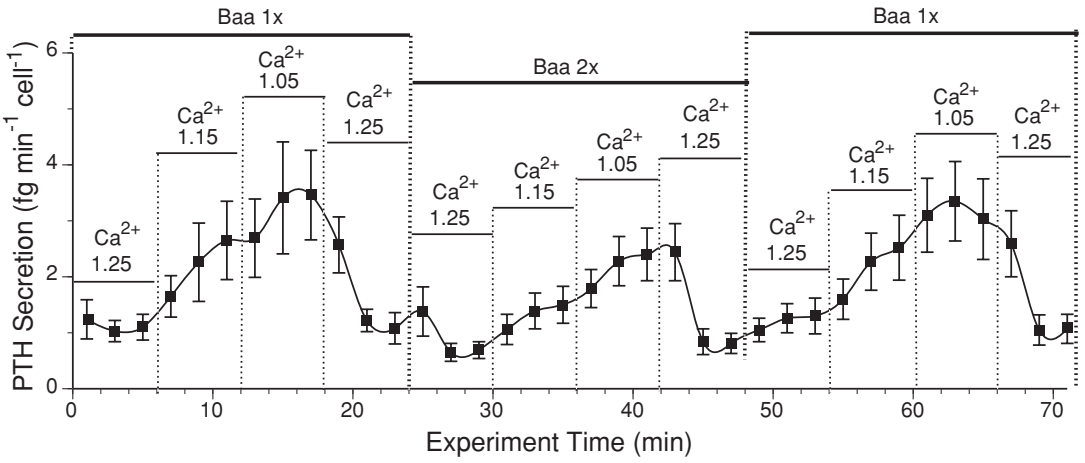

Figure 3

Impact of an increase in the fold concentration of a plasma-like amino acid mixture on parathyroid hormone (PTH) secretion. Normal human parathyroid cells were perifused with physiological saline solutions containing a 1x plasma-like L-amino acid mixture and 1 mg/ml bovine serum albumin. The effect of an elevation in the fold concentration of the amino acid mixture from 1x to 2x that raises the total serum amino acid concentration from 2.8–5.6 mM is shown. The ionized $Ca^{2+}{}_o$ concentration range (1.05 mM–1.25 mM) encompasses the normal physiological range. Baa, basal amino acid mixture. Figure reproduced from Reference 47.

L-Trp, the most potent amino acid activators of the CaR, stimulate both gastric acid secretion and gastrin release (44), and amino acid–activated gastric acid release is dependent upon a threshold Ca^{2+} concentration of about 1.0 mM and is powerfully enhanced in response to increases in extracellular fluid Ca^{2+} concentration from 1.0–2.0 mM (27), a recognized feature of the amino acid–activated CaR (48). Acidification of the luminal contents promotes calcium absorption—most probably by solubilizing calcium salts to release the ionized species Ca^{2+}.

Activation of CaRs in the parathyroid and thyroid C-cells result in suppressed PTH secretion and stimulated release of calcitonin, respectively (reviewed in 24). Both these events have the potential to promote bone mass by reducing PTH-induced osteoblast-dependent activation of osteoclasts and via calcitonin receptors on osteoclasts that suppress bone resorption. Recent evidence indicates that elevated amino acid levels do indeed suppress PTH release from normal human parathyroid cells in vitro (**Figure 3**) and that CaR-active amino acids including L-Phe, L-Trp, L-His, and L-Ala are more potent than other amino acids (47). In addition, preliminary data from one of us (A. Conigrave, unpublished findings) support the conclusion that amino acids activate CaRs in human C-cells.

High-protein diets (6, 80, 98) and amino acids infused intravenously (11, 118, 126) stimulate renal calcium excretion, findings that suggest a molecular and cellular link between an elevation in plasma amino acid levels and the control of renal Ca^{2+} filtration and/or reabsorption. As noted above, the Ca^{2+}-activated CaR promotes renal calcium excretion by (*a*) suppressing PTH release by CaRs expressed by parathyroid cells and (*b*) attenuating Ca^{2+} reabsorption by the action of CaRs expressed in the CTAL. Thus, amino acid activation of CaRs could explain high dietary protein–induced hypercalciuria via effects on the parathyroid or kidney. Alternatively, it might arise secondary to enhanced intestinal calcium absorption. Indeed, a recent analysis in pre- and postmenopausal women demonstrates that more than 90% of the protein-induced increase in renal calcium excretion arises from enhanced intestinal calcium absorption (102). Preliminary

evidence suggests that intravenous infusions of the CaR-active amino acid L-Phe, as well as the calcimimetic NPS R467, induce prompt and reversible increases in renal calcium excretion in rats (46). In addition, dietary supplementation with CaR-active aromatic amino acids was recently reported to promote intestinal calcium absorption and renal calcium excretion in humans (52).

It is currently unknown whether amino acid activation of the CaR might contribute to the positive impact of dietary protein on bone mass via CaRs expressed in osteoblast progenitors, osteoblasts or osteoclasts.

ROLES OF OTHER AMINO ACID–SENSING CLASS 3 GPCRS IN THE CONTROL OF CALCIUM METABOLISM

Currently, the roles of other class 3 GPCRs in bone and mineral metabolism are poorly defined. GPRC6A exhibits low-potency sensitivity to Ca^{2+} ions when expressed in HEK293 cells and is expressed in bone cells including osteoblastic cells (136). Furthermore, a preliminary report indicates that a GPRC6A-null mouse exhibits an osteopenic phenotype (138), and the GPRC6A amino acid activators arginine and lysine promote osteoblast-dependent production of IGF-1 and collagen synthesis in vitro (39). These interesting observations raise the possibility that two closely related amino acid–sensing class 3 GPCRs, GPRC6A and the CaR, may operate together in support of osteoblast differentiation, number, and cell function, thereby promoting bone mass and mineralization. Putative roles for the CaR and GPRC6A in the control of calcium metabolism and bone homeostasis are presented in **Table 1**.

CONCLUSIONS

Moderate-to-high dietary protein intake has positive effects on bone health, most obviously increased bone growth and peak bone mass in children and increased bone mineral density and a reduced rate of bone loss in adults. The impact of dietary protein intake on fracture incidence has been less certain, but several recent studies have concluded that dietary protein reduces the risk of hip fractures; other studies demonstrate that protein supplements reduce complications in the recovery phase following a hip fracture. The mechanisms that underlie the effects of protein on bone homeostasis are only now emerging. They include mechanisms that link changes in amino acid levels to the control of calcium absorption and excretion, effects on the hormonal milieu including elevated levels of IGF1 and suppressed levels of PTH, and effects on the fate and function of bone cells. One important group of amino acid sensors belongs to GPCR class 3, which includes the calcium-sensing receptor, a key regulator of calcium homeostasis and a modulator of bone metabolism.

DISCLOSURE STATEMENT

E.M. Brown has a financial interest in the calcimimetic, sensipar (cinacalcet HCl).

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Errata

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