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Extracellular Calcium Sensing and Extracellular Calcium Signaling

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Brown, Edward M., and R. John MacLeod. Extracellular Calcium Sensing and Extracellular Calcium Signaling. *Physiol Rev* 81: 239–297, 2001.—The cloning of a G protein-coupled extracellular Ca^{2+} (Ca_o^{2+})-sensing receptor (CaR) has elucidated the molecular basis for many of the previously recognized effects of Ca_o^{2+} on tissues that maintain systemic Ca_o^{2+} homeostasis, especially parathyroid chief cells and several cells in the kidney. The availability of the cloned CaR enabled the development of DNA and antibody probes for identifying the CaR's mRNA and protein, respectively, within these and other tissues. It also permitted the identification of human diseases resulting from inactivating or activating mutations of the CaR gene and the subsequent generation of mice with targeted disruption of the CaR gene. The characteristic alterations in parathyroid and renal function in these patients and in the mice with "knockout" of the CaR gene have provided valuable information on the CaR's physiological roles in these tissues participating in mineral ion homeostasis. Nevertheless, relatively little is known about how the CaR regulates other tissues involved in systemic Ca_o^{2+} homeostasis, particularly bone and intestine. Moreover, there is evidence that additional Ca_o^{2+} sensors may exist in bone cells that mediate some or even all of the known effects of Ca_o^{2+} on these cells. Even more remains to be learned about the CaR's function in the rapidly growing list of cells that express it but are uninvolved in systemic Ca_o^{2+} metabolism. Available data suggest that the receptor serves numerous roles outside of systemic mineral ion homeostasis, ranging from the regulation of hormonal secretion and the activities of various ion channels to the longer term control of gene expression, programmed cell death (apoptosis), and cellular proliferation. In some cases, the CaR on these "nonhomeostatic" cells responds to local changes in Ca_o^{2+} taking place within compartments of the extracellular fluid (ECF) that communicate with the outside environment (e.g., the gastrointestinal tract). In others, localized changes in Ca_o^{2+} within the ECF can originate from several mechanisms, including fluxes of calcium ions into or out of cellular or extracellular stores or across epithelium that absorb or secrete Ca^{2+}. In any event, the CaR and other receptors/sensors for Ca_o^{2+} and probably for other extracellular ions represent versatile regulators of numerous cellular functions and may serve as important therapeutic targets.

I. INTRODUCTION

A great deal has occurred in the field of extracellular Ca^{2+} (Ca_o^{2+}) sensing since 1991 when an earlier article in *Physiological Reviews* addressed this subject (52). At that time it was apparent that certain cells, such as the chief cells of the parathyroid gland, were capable of sensing (i.e., recognizing and responding to) small changes in the extracellular ionized calcium concentration. Moreover, indirect evidence suggested that Ca_o^{2+} sensing by parathyroid cells involved a process sharing certain properties with the mechanism through which G protein-coupled, cell surface receptors for a variety of extracellular messengers (e.g., peptides, catecholamines, prostaglandins) responded to their respective agonists (48, 95, 220, 315, 409). The cloning of a G protein-coupled Ca_o^{2+}-sensing receptor (CaR) from bovine parathyroid gland in 1993 (58) proved that the calcium ion can, in fact, serve as an extracellular first messenger.[1]

This review addresses the following areas in which progress has been particularly rapid over the past 5–10 years in elucidating the mechanisms underlying Ca_o^{2+}

[1] In this review we refer to the Ca_o^{2+}-sensing receptor originally cloned from bovine parathyroid by the abbreviation CaR; in some cases, the alternative designation CaSR (214) has been employed, while the abbreviation CaS, for calcium sensor, has been used to describe another putative Ca_o^{2+}-sensing protein with an entirely distinct structure (273) (see sect. IV*B*).

sensing. First, we briefly review the cloning of the CaR from various cells expressing it and discuss what is known about its homology with other members of the superfamily of G protein-coupled receptors (GPCRs), including some that also sense Ca_o^{2+}. Next we describe what is known about the structure of the CaR gene and the factors regulating its expression and review the results of recent studies on the receptor's structure-function relationships and the various intracellular signaling pathways to which it couples. The discussion then covers the rapidly expanding range of cellular functions regulated by the CaR, including its physiological roles in the cells expressing it that are involved in as well as those that are uninvolved in systemic mineral ion homeostasis. Finally, we address the related area of Ca_o^{2+} signaling, that is, the mechanisms that underlie local and/or systemic changes in Ca_o^{2+}, thereby producing signals that can modulate the receptor's activity both in tissues involved in systemic Ca_o^{2+} homeostasis as well as those that are not. We do not address disorders of Ca_o^{2+} sensing resulting from abnormalities in the CaR's structure and/or function (for review, see Ref. 54), except insofar as they elucidate the CaR's role of normal physiology.

II. MOLECULAR CLONING OF PARATHYROID, RENAL, AND OTHER EXTRACELLULAR CALCIUM-SENSING RECEPTORS

Expression cloning in *Xenopus laevis* oocytes enabled isolation of a single 5.3-kb clone (BoPCaR = bovine parathyroid Ca_o^{2+}-sensing receptor) that exhibited pharmacological properties very similar to those of the Ca_o^{2+}-sensing mechanism expressed endogenously in bovine parathyroid cells (58, 96, 368). Nucleic acid hybridization-based techniques then led to the cloning of full-length CaRs from several different tissues in various mammalian species, including human parathyroid (157); rat (381), human (3), and rabbit kidney (71); rat C cells (146, 158); and striatum of rat brain (392). All are highly homologous (>90% identical in their amino acid sequences to BoPCaR) and represent species and tissue homologs of the same ancestral gene.

A full-length CaR has also been cloned and characterized from chicken parathyroid (126), and a smaller segment of the CaR has been amplified and sequenced by RT-PCR from gastric mucosa of the mudpuppy, an amphibian (104). The chicken CaR and the portion of the mudpuppy CaR available for analysis exhibit slightly lower but still very substantial levels of homology to mammalian CaRs (84% identity at the amino acid level for the chicken CaR and 84% identity at the nucleotide level for the mudpuppy CaR), stressing the high degree of conservation of this gene among members of the mammals, birds, and amphibians examined to date.

Mammals, birds, amphibians, and reptiles, the so-called tetrapods, e.g., organisms having four extremities, all possess parathyroid glands and utilize Ca_o^{2+} homeostatic mechanisms similar in their overall design (335). Not surprisingly perhaps, given its wide tissue distribution, particularly in tissues apparently uninvolved in systemic mineral ion metabolism (see sect. XIII), the CaR did not originate when the parathyroid gland first appeared during evolution. A highly homologous CaR gene has been identified in fishes and in the dogfish shark (26). These species have levels of Ca_o^{2+} in their blood and extracellular fluids that are not dissimilar from those in humans and other mammals. They utilize hormones distinct from parathyroid hormone (PTH), such as stanniocalcin in fishes, for example (446, 447), to maintain Ca_o^{2+} homeostasis. Little work on the CaR's role in mineral ion homeostasis is available in these species. Further work is needed to determine whether, similar to its role in tetrapods, the CaR in these aquatic species controls Ca_o^{2+} both by regulating the secretion of calciotropic hormones, which then modulate the functions of target tissues (e.g., kidney, intestine, and gill), and by exerting direct, CaR-mediated actions on mineral ion transport by the latter. Given the crucial roles that both extra- and intracellular calcium play in essentially all organisms, it will be of great interest in future studies to understand the ontogeny and phylogeny of the CaR over a much broader evolutionary scale.

Surprisingly, given the diversity of structurally related GPCRs (see sect. III), an extensive search has yet to uncover definitive evidence for additional CaR isoforms arising from distinct genes, although there are several splice variants of the receptor that are expressed in various tissues (see sect. V*C*). The latter are currently of uncertain physiological relevance. Furthermore, there may be additional, physiologically relevant Ca_o^{2+} sensors, which are described in section IV.

The amino acid sequences of BoPCaR and the other CaRs cloned to date that are predicted from their nucleotide sequences reveal a common overall topology, which includes a very large (~600 amino acids), NH_2-terminal extracellular domain (ECD), a central core of some 250 amino acids with seven predicted transmembrane domains (TMDs) that are characteristic of the superfamily of GPCRs and a large intracellular COOH-terminal tail of ~200 amino acids (58) (Fig. 1). As described in more detail in section VI*D*, data from studies on the CaR's structure-function relationships indicate that Ca_o^{2+} binds to its ECD. The receptor's ECD also contains multiple *N*-linked glycosylation sites (58, 134), whereas its intracellular domains [three intracellular loops (ICLs) and COOH tail] harbor several predicted consensus protein kinase C (PKC) and protein kinase A (PKA) phosphorylation sites (the PKA sites are present in all species studied to date except the bovine CaR). The PKC phosphorylation sites are known to modulate the receptor's activity,

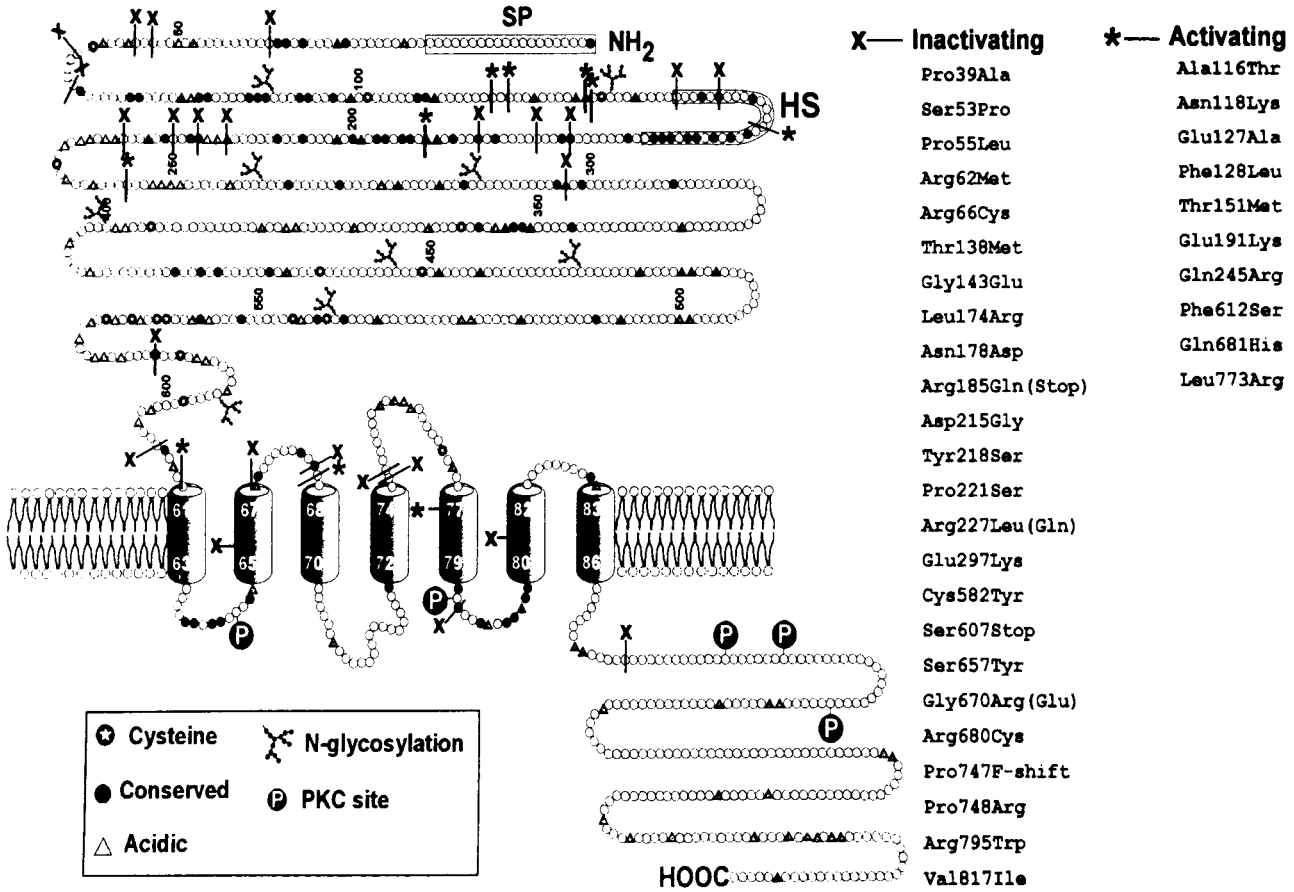

FIG. 1. Schematic representation of the principal predicted topological features of the extracellular Ca^{2+}-sensing receptor (CaR) cloned from human parathyroid gland. SP, signal peptide; HS, hydrophobic segment; PKC, protein kinase C. Also delineated are missense and nonsense mutations causing either familial hypocalciuric hypercalcemia or autosomal dominant hypocalcemia. These are indicated using the three-letter amino acid code, with the normal amino acid indicated before and the mutated amino acid shown after the numbers of the relevant codons. [From Brown EM, Bai M, and Pollak M. Familial benign hypocalciuric hypercalcemia and other syndromes of altered responsiveness to extracellular calcium. In: *Metabolic Bone Diseases* (3rd ed.), edited by Krane SM and Avioli LV. San Diego, CA: Academic, 1997, p. 479–499.]

whereas the physiological relevance of the PKA sites, if any, is currently unknown (18, 58, 89) (see sect. VI*F*).

III. MOLECULAR SIMILARITY OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR TO OTHER G PROTEIN-COUPLED RECEPTORS

Based on the evolutionary tree predicted from the database for GPCRs [GCRD; http://www.uthscsa.edu (251)], the CaR belongs to the recently described family C within this large superfamily of genes. Family C GPCRs are defined as a group of receptors comprising at least three different subfamilies that share ≥20% amino acid identity over their seven membrane-spanning region (251) (Fig. 2). Group I includes the metabotropic glutamate receptors, mGluRs 1–8, which are receptors for the excitatory neurotransmitter glutamate and are widely expressed in the central nervous system (CNS) (311, 312). Unlike the ionotropic glutamate receptors (iGluRs) [i.e., the *N*-methyl-D-aspartate (NMDA) receptor], which are ion channels containing a binding site for their physiological agonist glutamate within the same channel molecule, the mGluRs are GPCRs.

Group II contains at least two types of receptors: the CaR and a recently discovered, multigene subfamily of putative pheromone receptors, VRs (vomeronasal receptors) or GoVNs (193, 286, 397). The latter are found exclusively in neurons of the vomeronasal organ of the rat (VNO; hence the VRs) (a small sensory organ thought to be involved in regulating instinctual behavior through input from environmental pheromones) that express the guanine regulatory (G) protein, Ga_o (hence the GoVNs) (286). Additional receptors closely related to the CaR and/or VRs have recently been identified in mammals (203) and fishes (74, 310), which are taste and putative

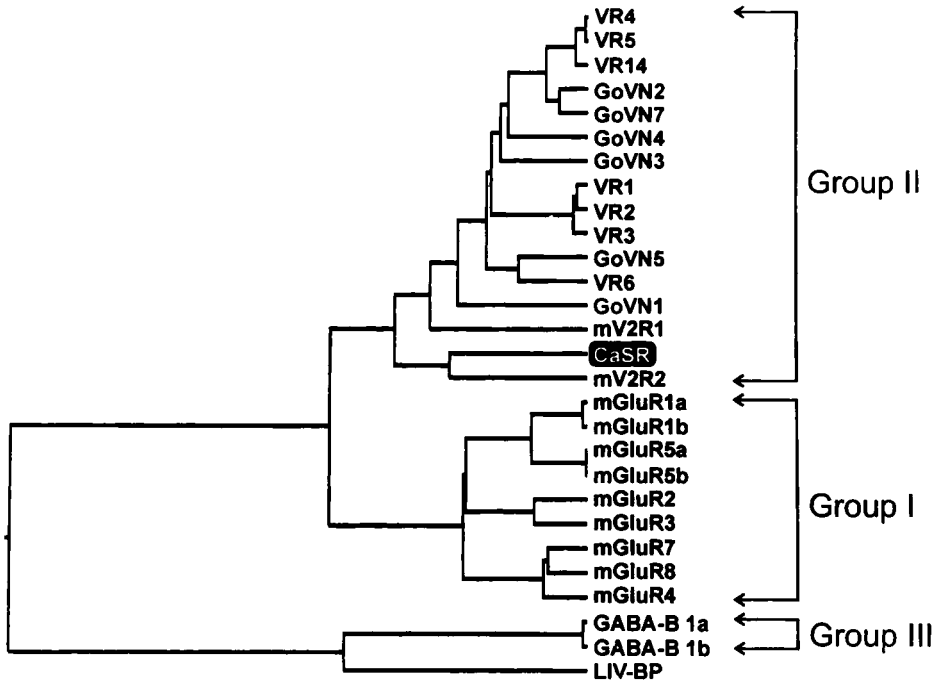

FIG. 2. "Tree" diagram showing the degrees of homology and proposed evolutionary relationships among the various members of the family C G protein-coupled receptors (GPCRs) described to date. The farther to the left that a given receptor branches off, the less related it is to the other receptors. For details see text. [From Brown et al. (61).]

odorant receptors, respectively. These related receptors in fishes may represent evolutionary precursors of the pheromone receptors in terrestrial organisms (e.g., rats); they exhibit the topology characteristic of the family C GPCRs and are most closely related to the CaR among the members of this family.

Group III contains a subfamily of receptors, the $GABA_B$ receptors, that bind and are activated by the inhibitory neurotransmitter GABA (238). As with the receptors for glutamate, there are both G protein-coupled and ionotropic (e.g., ligand-gated receptor channels) receptors for GABA (the latter are known as $GABA_A$ receptors) (458). Interestingly, the formation of a functional $GABA_B$ receptor capable of activating an inwardly rectifying K^+ channel requires heterodimerization of the two different members of this subfamily of receptors identified to date [e.g., $GABA_BR1$ (of which there are two splice variants: $GABA_BR1a$ and $GABA_BR1b$) and $GABA_BR2$], while homodimers of the individual $GABA_B$ receptor subtypes do not activate this ion channel (218, 239, 257, 456). The $GABA_BR2$ form of this receptor, however, can inhibit adenylate cyclase when expressed by itself in heterologous mammalian expression systems (257). As described in more detail in sections VI*A* and VI*B*, the CaR resides on the cell surface (16) and in detergent extracts of at least some CaR-expressing cells (452) as a dimer [e.g., in bovine parathyroid chief cells, the epithelial cells of the inner medullary collecting duct (IMCD) of the rat kidney, and in CaR-transfected human embryonic kidney (HEK293) cells]. Furthermore, there appear to be functional interactions between the individual monomeric subunits within these dimers, as detailed in section VI*E* (17).

The extracellular, ligand-binding domains of the family C GPCRs are structurally related to those of the bacterial periplasmic binding proteins (PBPs) (110, 329). This hypothesis was initially based on molecular modeling (330, 428) using the known clawlike, three-dimensional structure of the PBPs (329). More recent studies have identified a significant degree of sequence homology when comparing the ECDs of the $GABA_B$ receptor and the leucine-isoleucine-valine (LIV) bacterial nutrient-binding protein (238). This observation adds strong support to the hypothesis that there is an evolutionary link between the ECD of the various members of the family C GPCRs and the bacterial periplasmic, nutrient-binding proteins. Furthermore, recent data indicate that homologous regions of the ECDs of the $GABA_B$ receptors, mGluRs, and CaR may participate in the binding of GABA, glutamate, and Ca_o^{2+} (41), respectively, while a nearby but spatially distinct region of the ECDs of some mGluRs participates in the modulation of these latter receptors' activities by Ca_o^{2+} (256) (see sect. IV*A*).

The bacterial PBPs include at least eight families that recognize a broad range of extracellular solutes, which are destined for cellular uptake and/or elicit chemotactic responses (407, 428). These solutes include organic nutrients as well as inorganic ions, e.g., phosphate and nickel (407, 428). Interestingly, one of the PBPs, PhoQ, a gene expressed by *Salmonella*, is an extracellular Mg^{2+} (Mg_o^{2+}) sensor. PhoQ induces bacterial production of Mg^{2+} trans-

port proteins in response to environmental Mg^{2+} deprivation (299).

In their capacities to act as cell surface receptors participating in chemoreception and sensory transduction or membrane transport, the PBPs interact with integral membrane proteins within the bacterial cell membrane after they bind specific chemosensory substances or nutrients to transmit the signal or transport the nutrient into the cell. Therefore, it seems likely that the family C GPCRs, including the CaR, evolved as "fusion proteins" comprising an NH_2-terminal ECD derived from an ancient family of solute-binding proteins and the seven membrane-spanning, "serpentine" motif that evolved separately to transmit extracellular signals to the interior of eukaryotic cells via the GPCRs. Interestingly, the CaR can also participate in the stimulation of chemotaxis by Ca_o^{2+} in monocytes (464) and, perhaps, osteoblasts and their precursors (462, 463). Thus there may be conservation of both the functional as well as the structural attributes of this domain across a very broad evolutionary time scale.

IV. ARE THERE ADDITIONAL EXTRACELLULAR CALCIUM-SENSING RECEPTORS OR SENSORS?

A. mGluRs and $GABA_B$ Receptors

Recent work has revealed that some mGluRs can sense Ca_o^{2+} in addition to responding to glutamate as their principal agonist in vivo, although the physiological relevance of this Ca_o^{2+}-sensing remains uncertain. Kubo et al. (256) demonstrated that mGluRs 1, 3, and 5 sense Ca_o^{2+} over a range of ~0.1–10 mM, while mGluR2 is considerably less responsive to changes in Ca_o^{2+}. Construction of chimeric receptors, in which the ECDs of mGluR2 and -3 were fused to the TMDs and COOH tail of mGluR1a, proved that the capacities of the respective receptors to be activated by Ca_o^{2+} (or lack thereof) was conferred by their ECDs. All three of the mGluRs that sense Ca_o^{2+} have identical serines and threonines, respectively, at amino acid positions homologous to residues 165 and 188 in mGluR1a (41). These two residues are thought to play key roles in the binding of glutamate to the ECDs of the mGluRs (330). In contrast, while mGluRs 1a, 3, and 5 have a serine at a position equivalent to residue 166 in mGluR1a, mGluR2 has an aspartate in this position (256). Furthermore, changing the serines in mGluRs 1a, 3, and 5 to an aspartate considerably reduces their capacity to sense Ca_o^{2+}, while replacing the aspartate in mGluR2 with a serine increases its apparent affinity for Ca_o^{2+}, to a level similar to those of mGluRs 1a, 3, and 5 (256). Therefore, the serines at amino acid position 166 in mGluR1a and at the equivalent positions in mGluRs 3 and 5 apparently play key roles in their capacities to sense Ca_o^{2+}, although the molecular mechanism underlying this action is not clear from these studies. It should be pointed out, however, that the Hill coefficients for the modulation of the activities of these mGluRs by Ca_o^{2+} (as well as by glutamate) are close to one (256), considerably lower than that for the CaR, which is ~3 (15, 52, 58, 152, 393). Thus there are apparently additional aspects of the binding of Ca_o^{2+} by the CaR (e.g., the presence of several binding sites) and/or subsequent steps in its activation that confer positive cooperativity on this overall process (see sect. VI). The latter is a key element contributing to the narrow range within which Ca_o^{2+} is maintained by the mineral ion homeostatic system.

Of interest, a recent study has documented that changes in Ca_o^{2+} also modulate the $GABA_B$ receptors, although Ca_o^{2+} by itself has no effect on this class of receptors (459). Ca_o^{2+} potentiated the stimulatory action of GABA on GTP binding to this receptor and enhanced the coupling of the $GABA_B$ receptor to activation of a K^+ channel and inhibition of forskolin-stimulated cAMP accumulation. The actions of Ca_o^{2+} were not mimicked by other polyvalent cations. Therefore, given that not only Ca_o^{2+}, but also amino acids (109; see also sect. IX*E*), modulate the function of the CaR, when taken in the context of the actions of amino acids (e.g., glutamate) or their derivatives (i.e., GABA) on the mGluRs and $GABA_B$ receptors, respectively, further emphasizes the structural and functional relationships among these three types of receptors.

B. Other Putative Ca_o^{2+} Sensors

It is likely that there are Ca_o^{2+} receptors or sensors in addition to the CaR and mGluRs, which mediate some of the very substantial number of actions of Ca_o^{2+} on diverse cell types. The availability of the cloned CaR has made it feasible to begin to catalog the cell types that express this receptor, as described in more detail in sections X, XI, and XIII. For example, the inhibitory action of Ca_o^{2+} on PTH secretion, the stimulatory effect of Ca_o^{2+} on calcitonin (CT) release (146, 158, 288) and many of the actions of Ca_o^{2+} on the kidney (for review, see Ref. 180) are most likely CaR mediated. The presence of the CaR in a cell whose function is modulated by Ca_o^{2+} does not prove, however, that it mediates that particular action of Ca_o^{2+}. The availability of mice with targeted disruption of the CaR gene (196) and the discovery of human diseases caused by CaR mutations (for review, see Ref. 53) have provided very useful tools for assessing this receptor's role in Ca_o^{2+}-induced changes in various cellular functions in vivo and/or in vitro. The recent development of selective activators (320) and antagonists of the CaR (318) as well as the use of dominant negative CaR constructs (14, 15, 291) will likewise be of utility in determining whether

the CaR mediates known actions of Ca_o^{2+} on specific, CaR-expressing cell types.

There are, however, cells whose functions are modulated by Ca_o^{2+} that do not express the CaR or have not yet been examined for its expression. In the former case, the actions of Ca_o^{2+} could potentially be mediated by one or more of the mGluRs that sense Ca_o^{2+}, an hypothesis that has not yet been tested. Alternatively, this Ca_o^{2+}-sensing capability may be conferred by one or more of the additional putative Ca_o^{2+} receptors/sensors discussed below. There may well be other Ca_o^{2+}-receptors/sensing mechanisms as well, although a discussion of the evidence supporting the existence of these is beyond the scope of this review.

1. Megalin/gp330

Monoclonal antibodies that are directed at a large protein, called megalin or gp330, that is present at high levels in parathyroid, proximal tubular, and placental cells (220) as well as in a variety of other cell types can modulate the Ca_o^{2+}-sensing functions of these cells (223). For instance, such antibodies can interfere with the capacity of high Ca_o^{2+} to inhibit PTH secretion from human parathyroid cells (221). The level of expression of this protein is reduced substantially in pathological parathyroid glands from patients having various forms of hyperparathyroidism (HPT) (222). In these hyperparathyroid states, the abnormal cells are generally less sensitive than normal parathyroid cells to the suppressive effect of high Ca_o^{2+} on PTH release (51, 170, 326). Therefore, the reduced expression of the protein recognized by these antibodies could conceivably contribute to the defective Ca_o^{2+} sensing in HPT. Furthermore, the same protein could potentially participate in Ca_o^{2+} sensing by normal parathyroid cells. The level of expression of the CaR in pathological parathyroid cells, however, has also been found to be reduced in HPT in most (136, 162, 246) but not all studies (155), raising the possibility that the changes in megalin expression in hyperparathyroidism could be the consequence rather than the cause of the disease.

cDNAs coding for megalin/gp330 have been isolated from human (195, 273) and rat cDNA libraries (398). These cDNAs encode very large, ~500-kDa proteins that belong to the low-density lipoprotein receptor superfamily. Recent studies have provided strong evidence that megalin's principal role is to serve as an endocytic receptor (116) that binds to and mediates uptake of albumin (115), insulin (333), the transcobalamin-B12 complex (101), retinol and its binding protein (103), and thyroglobulin (279), as well as other proteins (298) and even drugs (137). Indeed, megalin "knockout" mice show defective proximal tubular uptake of the serum vitamin D and retinol binding proteins and their associated vitamin D metabolites and retinol, respectively, providing strong support for the role of this protein as an endocytic receptor (103). Although megalin does bind extracellular calcium ions (102), this Ca_o^{2+} binding probably does not participate directly in systemic mineral ion metabolism. It will be of interest to determine whether megalin interacts with the CaR in cells that coexpress both proteins and/or regulates the CaR's internalization or other aspects of its function, thereby participating indirectly in Ca_o^{2+} sensing.

2. Ca_o^{2+} sensing by osteoblasts

Raising Ca_o^{2+} has several actions on cells of the osteoblastic lineage. Elevated levels of Ca_o^{2+} stimulate bone formation in explants of rodent bone (372). In addition, Ca_o^{2+} and other polycations [e.g., strontium (73) and aluminum (Al^{3+}) (364)] stimulate the proliferation (161, 363, 421) and/or chemotaxis (161) of osteoblasts and their precursors, an effect that could be mediated, in part, by an associated increase in the release of insulin-like growth factor II (IGF-II) (201). High Ca_o^{2+} also modulates intracellular second messengers in the murine osteoblastic cell line, MC3T3-E1. Elevated levels of Ca_o^{2+} raise diacylglycerol (174) and cAMP levels (175) in these cells but do not promote the formation of inositol phosphates that would occur with activation of phosphoinositide (PI)-specific phospholipase (PL) C. Quarles et al. (362) were unable to detect CaR transcripts by RT-PCR and Northern analysis in MC3T3-E1 cells and suggested on the basis of this result as well as pharmacological differences from the CaR [including the latter's low affinity for Al_o^{3+} (417)] that a distinct Ca_o^{2+}-sensing receptor mediated the actions of Ca_o^{2+} on this cell line. The same group has identified genomic clones for several CaR-related genes (194) that are ~60% similar and 40% identical to the CaR originally cloned from parathyroid (58) and kidney (381) within regions corresponding to their predicted TMDs. Transcripts for these putative receptors, however, are not expressed in bone cells at levels that can be detected by Northern analysis or RNase protection (194). It is possible, therefore, that they encode either pseudogenes or related receptors, viz., homologs of the putative pheromone receptors in the VNO of the rat (193, 286, 397) but are not involved in sensing Ca_o^{2+} and other polyvalent cations in osteoblasts. Moreover, as discussed in more detail in section x*D*, we (463) and others (230) have recently found that MC3T3-E1 cells express both CaR transcripts as assessed by RT-PCR and Northern analysis and receptor protein as detected by Western analysis and/or immunocytochemistry. Further studies of these and other osteoblastic cell lines are needed in which the CaR has been "knocked out" through the use of selective CaR activators (320) or antagonists (318) and/or dominant negative constructs of the CaR (291), to prove which, if any, of the effects of Ca_o^{2+} on osteoblastic cell lines are mediated by the CaR versus some other Ca_o^{2+}-sensing

mechanism(s). Furthermore, while MC3T3-E1 cells and other osteoblast-like cell lines represent useful models for investigating the control of osteoblastic function, they may or may not faithfully reproduce the phenotype of osteoblasts in vivo. It will be important, therefore, to determine whether bona fide osteoblasts and/or their precursor cells in intact bone express the CaR and/or other Ca_o^{2+}-sensing mechanisms. Of interest in this regard, the studies of Pi et al. (349) have recently shown that primary osteoblasts derived from mice with targeted disruption of the CaR gene retain certain responses to Ca_o^{2+}, consistent with the presence of another Ca_o^{2+}-sensing mechanism (349). The presence of the latter and/or the CaR in osteoblasts could enable these cells to respond in physiologically relevant ways to local changes in Ca_o^{2+} within the bone/bone marrow microenvironment (see also sects. x*D* and XIV for further discussions of local Ca_o^{2+} sensing and Ca_o^{2+} signaling in bone, respectively).

3. Ca_o^{2+} sensing by osteoclasts

Another example of a cell that appears to possess a Ca_o^{2+}-sensing mechanism distinct from the CaR is the osteoclast, based largely on indirect, pharmacological evidence. Several groups first reported in 1989 that elevating Ca_o^{2+} had direct actions on isolated osteoclasts in vitro, inhibiting bone resorption and producing elevations in the cytosolic calcium concentration (Ca_i^{2+}), which were reminiscent of those elicited in parathyroid cells by raising Ca_o^{2+} (277, 476). Although it remains to be determined whether this mechanism functions in a physiologically relevant manner in vivo (e.g., by creating mice with targeted disruption of the relevant gene), it could represent a Ca_o^{2+}-sensing system through which the osteoclast regulates its own resorptive activity; that is, when Ca_o^{2+} rises above a certain level, owing to osteoclast-mediated bone resorption, activation of the putative Ca_o^{2+}-sensing receptor in this cell type would feed back to inhibit further bone breakdown. Subsequent studies, principally by Zaidi et al. (475), have elucidated several features of the process of Ca_o^{2+} sensing by osteoclasts (see below), although characterization of the sensor/receptor at a molecular level has not yet been accomplished.

Elevating Ca_o^{2+} in vitro produces marked retraction of osteoclasts, decreased expression of podosomes (the structures that anchor resorbing osteoclasts to the underlying bone), inhibition of the release of hydrolytic enzymes, and a reduction in bone resorption (277, 476). The observed Ca_o^{2+}-induced increases in Ca_i^{2+} are probably an important mediator of the associated alterations in cellular function, because the calcium ionophore ionomycin causes similar effects. Not all osteoclasts possess this Ca_o^{2+}-sensing mechanism. Those freshly isolated from medullary bone of the Japanese quail, for example, do not exhibit these responses to elevated levels of Ca_o^{2+} (25). After being cultured for 5–8 days, however, these cells develop the capacity to sense Ca_o^{2+} in a manner similar to that of osteoclasts freshly isolated from chick or rat bone (25). These cultured quail osteoclasts could, therefore, represent an appropriate source of mRNA encoding the putative sensor that could be utilized to isolate the relevant gene using an expression cloning strategy.

A variety of polyvalent cations mimic the actions of Ca_o^{2+} on the osteoclast, but they generally exhibit a pharmacological profile that differs distinctly from that exhibited by parathyroid cells and other cells expressing the CaR (405) [although more recent studies have provided examples of pharmacological profile more similar to that of the CaR, including effects of extracellular Gd^{3+} and neomycin resembling those of high Ca_o^{2+} (474); see also sect. x*D*]. In general, activation of the CaR in parathyroid cells by Ca_o^{2+}, Mg_o^{2+}, or extracellular Ba^{2+} takes place at concentrations of these divalent cations that are severalfold lower than those modulating the function of osteoclasts (57, 409, 477). The lower affinity of the Ca_o^{2+}-sensing mechanism in osteoclasts for Ca_o^{2+} may be physiologically appropriate, because Ca_o^{2+} measured directly beneath osteoclasts that are actively resorbing bone can be as high as 8–40 mM (412). Other polyvalent cations that activate the osteoclast's Ca_o^{2+}-sensing mechanism include extracellular Ni^{2+}, extracellular Cd^{2+} (which do not stimulate the CaR) (405), and extracellular La^{3+} (which does activate the CaR) (404).

The putative Ca_o^{2+}-sensing receptor in the osteoclast may be related to the ryanodine receptor (479). Agents [e.g., ryanodine (478) or caffeine (406)] that interact with and modulate the activity of the ryanodine receptor (which mediates high Ca_i^{2+}-induced release of Ca^{2+} from intracellular stores in skeletal muscle and other cell types) modify osteoclastic Ca_o^{2+} sensing. Moreover, osteoclasts bind [^{3}H]ryanodine, and this binding is displaced by Ca_o^{2+} and by the ryanodine receptor antagonist ruthenium red (479). Finally, an antibody that recognizes an epitope within the ryanodine receptor's channel-forming domain potentiates the effects of extracellular Ni^{2+} on osteoclasts and labels the plasma membrane of nonpermeabilized osteoclasts. Conversely, an antibody that interacts with an intracellular epitope does not exert either of these actions. Taken together, these results suggest the presence of a ryanodine receptor-like molecule on the osteoclast plasma membrane (479) (in contrast to other cell types in which the ryanodine receptor is located intracellularly) that functions as a Ca_o^{2+} sensor or in close association with some other Ca_o^{2+}-sensing mechanism. It should be pointed out, however (as described in more detail in sect. x*D*), that recent studies have suggested that the CaR is also expressed in osteoclasts and/or their precursors. It remains to be determined whether there are actually two distinct Ca_o^{2+}-sensing mechanisms in this cell type.

4. Genetic evidence for the existence of additional receptors/sensors

The identification of inherited diseases of Ca_o^{2+} sensing has not only provided strong genetic evidence for the central role of the CaR in systemic Ca_o^{2+} homeostasis but has also raised the possibility that there may be additional Ca_o^{2+} sensors/receptors. Familial hypocalciuric hypercalcemia (FHH) is a generally benign, inherited condition (indeed it is sometimes called FBH, familial benign hypercalcemia) in which there is autosomal dominant inheritance of hypercalcemia accompanied in most cases by relative hypocalciuria (i.e., lower rates of urinary calcium excretion than would have been expected in the setting of hypercalcemia) (for review, see Ref. 49). The great majority of families with this condition (at least 90%) show genetic linkage to the long arm of chromosome 3 in the region where the CaR gene is known to reside (100, 179, 353, 354, 434). Of the families exhibiting this linkage to chromosome 3, about two-thirds have heterozygous inactivating mutations within the coding region of the CaR gene (49). Most of these mutations are point mutations that reduce the receptor's activity by decreasing its cell surface expression and/or reducing its intrinsic biological activity. Some mutations exert an additional dominant negative action on the wild-type CaR (15, 178, 342, 353).

In a few families, consanguineous marriages of individuals with FHH (yielding infants homozygous for CaR inactivation) (213, 353, 354) or union of persons with FHH harboring different CaR mutations (producing a compound heterozygous infant) (249) produces a much more severe form of hypercalcemia, termed neonatal severe hyperparathyroidism (NSHPT) (for review, see Ref. 49). The discovery that FHH and NSHPT can represent, respectively, the equivalent of the heterozygous and homozygous forms of complete or partial knockout of the CaR gene has *1*) established the central, nonredundant role of the CaR in mineral ion metabolism, *2*) proved that expression of the CaR is required for normal regulation of PTH secretion and probably parathyroid cellular proliferation by Ca_o^{2+} (see also sect. x*A*), and *3*) documented that the CaR plays a key role in regulating the renal tubular handling of divalent cations (see sect. x*C* for more details).

Of great interest, clinical conditions similar in many of their features to FHH can be caused by genetic defects at chromosomal loci other than that harboring the CaR gene. The first such condition was assigned to a locus on chromosome 19p13.3 by Heath et al. (179) in a family with clinical characteristics indistinguishable from those present in the form of FHH caused by mutations in the CaR. Subsequently, Trump et al. (434) have shown that another family with FHH exhibiting certain atypical features (e.g., osteomalacia and progressive elevations in serum PTH with increasing age in certain family members) exhibits linkage to a different locus on chromosome 19 (19q13) (269), further documenting the genetic heterogeneity of this clinical syndrome. It is possible, therefore, that these two genetic loci contain genes encoding Ca_o^{2+} sensors other than the CaR. Alternatively, these genes might represent additional, presumably downstream elements along the Ca_o^{2+}-sensing pathway(s) regulated by the CaR (or some other Ca_o^{2+} sensor) that, when mutated, interfere with the ability of parathyroid and kidney to respond normally to the Ca_o^{2+} signal. It is also conceivable that these genes encode transcription factors or other proteins necessary for expression of the CaR gene in parathyroid and kidney. In the latter case, loss of the relevant transcription factor might reduce the expression of the CaR, analogous to certain forms of diabetes that result from mutations in transcription factors participating in expression of the insulin gene (165).

V. THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR GENE AND REGULATION OF EXTRACELLULAR CALCIUM-SENSING RECEPTOR EXPRESSION

A. The CaR Gene

Very little work has been carried out directed at characterizing the CaR gene. The human gene is located on the long arm of chromosome 3 (3q21-q24) as assessed by linkage analysis (100) and at band 3q13.3–21 as determined by fluorescent in situ hybridization (214). In the rat and mouse, the gene resides on chromosomes 11 and 16 (214), respectively, while in the bovine species it is present on chromosome 1 (314). The human CaR gene contains at least seven exons (343). Six encode the receptor's large ECD and/or its upstream untranslated regions, while a single exon codes for the receptor's TMDs and COOH terminus (343, 353). The regulatory regions of the gene have not yet been characterized but will be of substantial interest, since expression of the CaR can change under several circumstances in vivo and/or in vitro as described below.

B. Genetic Polymorphisms of the CaR

Three apparently benign polymorphisms have been identified in the predicted COOH tail of the CaR: A986S, G990R, Q1011E, which were present in 30, 15, and 10% of more than 100 persons in the United States who were apparently unaffected with any disturbance in calcium homeostasis (178). In a subsequent study (106), the A986S polymorphism was found to be present in 16% of 163 Canadian individuals and was associated with a slight increase in serum total calcium concentration when cor-

rected for albumin and in fasting calcium concentration (106). It is possible that this polymorphism could contribute to a genetic predisposition to certain bone and/or mineral disorders.

C. Splice Variants of the CaR

Several splice variants of the CaR gene have been described. One cDNA clone of the human parathyroid CaR contained a 30-nucleotide insertion within the region of the gene encoding the receptor's predicted ECD (157). This 10-amino acid insertion had no apparent effect on the function of the CaR as assessed by expression in *Xenopus laevis* oocytes (157). The same splice variant has subsequently been identified in human breast cancer tissue (97). Its functional significance, if any, when studied in mammalian expression systems requires further investigation. Another alternatively spliced CaR transcript that is expressed in human cytotrophoblasts and parathyroid lacks exon 3 and encodes a truncated, presumably inactive receptor (40). Whether this receptor could interfere in some way with the normal CaR's function or exhibits any other functional attribute(s) remains to be determined. Oda et al. (327) have recently described an alternatively spliced form of the CaR in keratinocytes that lacks exon 5, producing an in-frame deletion of 77 amino acids and resulting in an expressed protein that is smaller and exhibits an altered glycosylation pattern compared with the full-length CaR. The truncated CaR was inactive when transfected into HEK293 cells or keratinocytes as assessed by high Ca_o^{2+}-evoked increases in inositol phosphates, and it interfered with the function of the coexpressed full-length CaR (327). This latter observation may explain the reduced responsiveness of differentiated keratinocytes to Ca_o^{2+}-induced elevations of Ca_i^{2+}, as the alternatively spliced form of the receptor is expressed at greater levels in the differentiated cells and could exert, therefore, a dominant negative action on the full-length CaR expressed within the same cells (327).

Finally, there can be alternative splicing within the 5′-untranslated region (UTR) of the CaR gene. For instance, transcripts in human parathyroid vary within their 5′-UTRs, consistent with alternative splicing of noncoding exons within the 5′-upstream region of the gene, without, however, altering the coding region (157). Such alternative splicing within the gene's putative upstream regulatory regions could clearly participate in tissue-specific expression and/or regulation of the CaR gene, but further studies are needed to define further its importance in this regard. Chikatsu et al. (99) have recently cloned a portion of the upstream region of the human CaR gene and identified two promoters (present within, respectively, exons 1A and 1B). The more upstream of the two promoters has TATA and CAAT boxes, while the downstream promoter is GC rich.

D. Regulation of CaR Expression

1. CaR expression in cultured parathyroid cells

Recent studies have documented that the expression of the CaR mRNA and/or protein can change in a variety of circumstances, although the mechanisms underlying these alterations in gene expression are, as yet, poorly understood. Calf parathyroid cells show rapid (within hours and 1–2 days, respectively) and marked (up to 80–85%) reductions in CaR mRNA and protein after they are put in culture (47, 297). This reduction in CaR expression probably contributes to a major extent to the accompanying reduction in high Ca_o^{2+}-elicited inhibition of PTH release (47, 259, 260, 297). Of interest, the expression of the receptor and the associated suppression of PTH secretion by high Ca_o^{2+} are maintained to a substantially greater extent in long-term cultures of human parathyroid cells, for unclear reasons (391).

2. CaR expression in renal insufficiency

The level of expression of the CaR also decreases in the kidney in chronic renal insufficiency induced in rats by subtotal nephrectomy (283, 285). This reduction in CaR expression may contribute to the associated reduction in urinary Ca^{2+} excretion occurring in this setting, based on the inverse relationship between CaR activity and/or expression levels and concomitant renal excretion of Ca^{2+} that is present in persons with inactivating mutations in the CaR (see sect. x*C*) (181). Since, as described later, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] can increase the renal expression of the CaR (46), the decrease in CaR expression in the kidney with impaired renal function could result, at least in part, from the associated reduction in the level of 1,25$(OH)_2$D that occurs during the development of renal insufficiency (419). Alternatively, the rise in circulating PTH levels in the setting of chronic renal failure (419) may also contribute to the reduction in CaR gene expression in the kidney (285). Further studies are needed to distinguish between these possibilities.

3. Reduced CaR expression in hyperparathyroidism

The level of expression of the CaR in parathyroid cells is diminished in pathological parathyroid glands resected from patients with primary hyperparathyroidism or with the severe hyperparathyroidism that can develop during chronic hemodialysis in patients with renal failure due to end-stage renal disease (162, 246). This reduction in CaR mRNA and protein expression has been observed in most (45, 135, 136, 232) but not all studies (155). The study that did not show a decrease in receptor expression employed semi-quantitative RT-PCR to compare the levels of expression of CaR transcripts in normal and pathological parathyroid glands. One potential problem in the

interpretation of the results of this study (155) is that normal but not hyperparathyroid parathyroid glands contain substantial numbers of fat cells that can account for ~50% of the volume of the normal parathyroid gland. Studies using immunocytochemistry or in situ hybridization have not detected expression of the CaR in the fat cells of normal parathyroid glands (162, 246). Extraction of RNA from normal parathyroid glands (155), therefore, may have in effect "diluted" CaR transcripts from parathyroid chief cells with RNA not containing these transcripts from fats cells, thereby reducing apparent CaR mRNA expression in normal parathyroid glands to levels that are comparable to those in pathological parathyroid cells. A recent study has shown a selective reduction in parathyroid adenomas of the CaR transcript arising from the further upstream of the two promoters regulating this gene's expression (99). The mechanism underlying this change in the pattern of expression of the CaR gene, however, remains to be determined.

In view of the reduced sensitivity to Ca_o^{2+} of parathyroid glands from patients with inactivating mutations of the CaR gene, the observed reduction in CaR expression in pathological parathyroid tissue could contribute to the elevated set point for Ca_o^{2+}-regulated PTH secretion that is often observed not only in primary but also in severe, uremic secondary HPT (51, 62). The relationship of the reduced CaR expression to the associated excessive cellular proliferation in these pathological parathyroid glands remains to be determined. It should be noted, however, that the parathyroid cellular proliferation observed in mice with knockout of the CaR gene (196) as well as in patients homozygous for inactivating mutations of the human CaR gene (for review, see Ref. 49) strongly support the CaR's involvement in tonically suppressing parathyroid cellular proliferation. There is further discussion of the CaR's roles in controlling various aspects of parathyroid function in section x*A*.

4. *Effects of vitamin D and high Ca_o^{2+} on CaR expression*

There are interactions between the CaR (or at least the effects of high Ca_o^{2+}) and vitamin D receptor (VDR) that are likely physiologically relevant in that the two receptors regulate their own levels of expression and/or the expression of the other receptor. Vitamin D, specifically its active form $1,25(OH)_2D$, upregulates its own receptor at the transcriptional level (313). Vitamin D also increases the expression of the CaR in parathyroid and kidney in vivo in the rat (46), although another study, carried out using a slightly different experimental approach, failed to observe any vitamin D-induced changes in the CaR's expression in these two tissues (387). If confirmed in future studies, vitamin D-elicited upregulation of the CaR in the parathyroid could be physiologically appropriate, since it would tend to facilitate inhibition of parathyroid function by high Ca_o^{2+}, e.g., suppression of PTH secretion and parathyroid cellular proliferation (see sect. x*A*).

High Ca_o^{2+} raises the levels of expression of the CaR in the pituitary-derived, ACTH-secreting murine AtT-20 cell line (131) and of the VDR in rat parathyroid glands in vivo (394). Although these actions of Ca_o^{2+} have not yet been proven to be CaR mediated, taken together with the known effects of vitamin D on the expression of the CaR and VDR, they would afford the opportunity for synergistic interactions between Ca_o^{2+} and vitamin D in regulating their target tissues. Such interactions could contribute, for instance, to the known synergistic actions of vitamin D and Ca_o^{2+} in promoting the differentiation of the human colon cancer cell line Caco-2 (113) and in enhancing the expression of calbindin D_{28K} in the kidney (105). Furthermore, the combination of high Ca_o^{2+} and vitamin D could produce a synergistic inhibition of the expression of the preproPTH gene (413, 467), at least in part, by upregulating the receptors involved in mediating this action as well as through the direct effects of each agent on its own receptor. Further studies are needed, however, aimed at understanding the mechanism(s) (e.g., transcriptional or posttranscriptional) by which high Ca_o^{2+} and vitamin D regulate the expression of the CaR gene. Additional discussion of the regulation of the expression of various genes by high Ca_o^{2+} and the CaR can be found in section viii*D*.

Suzuki et al. (425) have recently identified thyroid transcription factor 1 (TTF-1) as a potentially important element in the mechanism(s) through which Ca_o^{2+} may induce changes in gene expression in CaR-expressing cells. TTF-1 is a transcription factor that is a key mediator of thyroid-specific gene expression. It is also expressed in thyroid C cells and in parathyroid chief cells and interacts with elements within the 5′-flanking regions of the CaR, calmodulin, and calcitonin genes (425). Increases or decreases in Ca_i^{2+} enhance or reduce, respectively, the activity of this promoter, its RNA levels, and the binding of TTF-1 to these genes. In CaR-expressing cells that also express TTF-1, in which activation of the receptor is linked to elevations in Ca_i^{2+}, therefore, TTF-1 may mediate the regulation of Ca_o^{2+}-dependent genes (425).

5. *High phosphorus intake and CaR expression*

Whether phosphorus intake modulates CaR expression is controversial. Brown et al. (45) showed that high phosphorus intake was associated with reduced CaR mRNA and protein expression in the parathyroid glands of rats with secondary hyperparathyroidism owing to subtotal nephrectomy. In another study using a similar model, in contrast, there was no change in CaR mRNA expression during intake of a high phosphorus diet (192). In the first of these studies, the reduced CaR immunostaining was limited to regions of active chief cell prolif-

eration, suggesting that the reduction in CaR expression might be secondary to enhanced proliferation rather than the change in phosphorus intake per se (45). It is possible that if high dietary phosphorus induces only focal changes in CaR expression, the latter might be missed by techniques that measure total (i.e., integrated) tissue levels of the receptor (e.g., Northern analysis) (192) as opposed to those that assess localized differences in expression (i.e., in situ hybridization or immunocytochemistry).

6. Developmental changes in CaR expression

There are substantial developmental increases in the expression of the CaR in both kidney (84, 88) and hippocampus of the rat (87). The upregulation of the CaR in the kidney occurs in the immediate peri- and postnatal period, and the ensuing higher level of expression of the receptor persists through adulthood (84, 88). The increase in CaR expression in brain, in contrast, occurs about a week postnatally. Furthermore, it is transient, decreasing severalfold ~2 wk later to a lower level that remains stable into adulthood (87). The mechanisms underlying these alterations in expression of the CaR gene, including the relative importance of alterations in gene transcription versus posttranscriptional mechanisms, require further investigation.

7. Other factors affecting CaR expression

Interleukin (IL)-1β modestly raises the level of CaR mRNA in bovine parathyroid gland fragments in association with a reduction in PTH secretion (324). In preliminary studies, we have also demonstrated that sheep that have undergone experimental burn injury show increased CaR mRNA and protein expression in parathyroid but not in kidney (E. D. Murphey, N. Chattopadhyay, M. Bai, O. Kifor, D. Harper, D. L. Traber, E. M. Brown, and G. L. Klein, unpublished data). Because the levels of inflammatory cytokines are elevated in patients who have suffered a burn, including IL-1 levels, the latter could potentially account, at least in part, for the accompanying rise in CaR expression in the sheep model of burn injury. Furthermore, the increase in CaR protein expression presumably contributes to the associated reduction in PTH secretion and to the failure of PTH levels to rise normally despite hypocalcemia that can occur after burn injury (248).

VI. STRUCTURE-FUNCTION RELATIONSHIPS OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR

A. Biochemical Evaluation of the CaR Expressed Endogenously and in CaR-Transfected Cells

CaR proteins extracted from HEK293 cells transiently transfected with the human CaR (15, 134) have similar expression patterns to those isolated from bovine parathyroid cells (15). Western blot analysis using anti-CaR antisera reveals a doublet of protein bands at molecular masses corresponding to ~130–140 and 150–160 kDa that are present in considerably greater amounts than another band at ~115–120 kDa (15, 134). The latter is close to, or slightly smaller than, the expected size of the full-length, nonglycosylated receptor protein predicted from the CaR's cDNA. Direct sequence analysis of the receptor's ECD, in fact, has revealed that the putative signal peptide at the CaR's NH_2 terminus predicted from its nucleotide sequence has been cleaved off (163). Thus the first residue encountered is the tyrosine predicted at amino acid position 20 (163) of the human CaR cDNA (157).

Biochemical analysis of CaR-transfected HEK293 cells using appropriate endoglycosidases has shown that the immunoreactive band at 130–140 kDa corresponds to an immature form(s) of the CaR glycosylated with carbohydrate(s) high in mannose content, while the band at 150–160 kDa is the mature form of the receptor glycosylated with complex carbohydrates (15, 134). The receptor present on the cell surface of these cells is the mature form of the receptor, although the latter probably only represents a relatively small fraction of the total cellular immunoreactivity of the CaR as assessed by Western blot analysis (16). Therefore, much of the CaR is located intracellularly. Indeed, immunocytochemistry using anti-CaR antisera performed on a variety of CaR-expressing cells, when performed following detergent permeabilization (e.g., Triton X-100), demonstrates substantial CaR immunoreactivity over the cytoplasm, often with a prominent perinuclear component (for review, see Ref. 88). It is not currently known whether intracellular forms of the CaR simply represent nascent receptor protein in passage through the biosynthetic pathway or whether these intracellular receptors have distinct biological functions. For instance, the concentration of Ca_o^{2+} within its intracellular stores in the endoplasmic reticulum (ER) (300) approaches the millimolar range and could potentially be sensed by the CaR's NH_2-terminal "extracellular" domain, which would face the lumen of the ER.

In addition to these monomeric forms of the CaR, there are variable amounts of a doublet of immunoreactivity on Western analysis performed using reducing agents at the expected molecular weights of high mannose-containing and fully glycosylated CaR dimers and, to a lesser extent, higher oligomers of the receptor (15, 452). These are not simply artifacts resulting from aggregation of the receptor occurring during its extraction from cells and subsequent PAGE in a denaturing buffer, since these dimers are also present when the receptor is extracted using nondenaturing buffers, and its size is estimated using gel permeation chromatography (452). Furthermore, Bai et al. (16) have shown using a nonpermeant

cross-linking reagent combined with cell surface biotinylation that most of the CaR on the cell surface of transiently transfected HEK293 cells is present as a dimer. These CaR dimers appear to be disulfide linked, as the inclusion of reducing agents, such as dithiothreitol or mercaptoethanol, is needed for their conversion to monomers (16, 452). Even following reduction, however, a substantial fraction of the receptor protein can still run on denaturing PAGE as a dimer (16). Therefore, there may be additional intermolecular interactions contributing to dimerization. Interestingly, the CaR has within its fifth TMD a putative hydrophobic dimerization motif present in one of the β_2-adrenergic receptor's TMDs (185). Moreover, the CaR is far from unique among the GPCRs in forming dimers. Recent studies have emphasized that a number of GPCRs (184, 219, 480), including the mGluRs (389), may exist as dimers and that dimerization may potentially play important roles in the function of these receptors.

B. Role of *N*-Linked Glycosylation in the CaR's Cell Surface Expression

As noted above, the CaR's mature, cell surface form has a carbohydrate content of ~35–40 kDa/receptor monomer as assessed on Western blots (15, 134). These carbohydrate residues could potentially contribute to the binding of Ca_o^{2+} or participate in other aspects of the receptor's structure and/or function. Indeed, treatment of CaR-transfected HEK293 cells with tunicamycin, which blocks *N*-linked glycosylation, markedly reduced the response of the cells to raised levels of Ca_o^{2+} in association with reduced cell surface expression of the receptor (134). The use of site-directed mutagenesis subsequently revealed that of the nine predicted *N*-liked glycosylation sites within the human CaR's ECD, eight are efficiently glycosylated (375). Removal of any four or five of these produced substantial (50–90%) reductions in cell surface expression and biological activity, and at least three intact glycosylation sites were required for efficient cell surface expression. Glycosylation per se, however, did not appear to be critical for the CaR's biological activity as assessed by high Ca_o^{2+}-induced increases in inositol phosphate accumulation (375).

C. Role of the CaR's ECD in Binding Ca_o^{2+}

The first direct evidence that Ca_o^{2+} binds to the CaR's ECD was provided by studies utilizing chimeric receptors in which the ECD of either the CaR or an mGluR was fused to the TMDs and COOH tail of the other receptor (317). A chimeric receptor containing the CaR's ECD and the TMDs and COOH tail of mGluR1a was activated by high Ca_o^{2+} but not by mGluR agonists. Conversely, a chimeric receptor comprising the mGluR's ECD and the CaR's TMDs and COOH tail was activated by glutamate but not by high Ca_o^{2+}. [In these studies, in which the receptors were expressed in *X. laevis* oocytes, activation of the mGluR by high Ca_o^{2+} was not observed (317); subsequent studies have shown, however, that certain mGluRs can sense Ca_o^{2+}, as noted above (256)]. Thus the ECD confers ligand specificity upon the CaR, the mGluRs, and presumably the other members of the family C GPCRs.

Subsequent studies (41) have confirmed that Ca_o^{2+} acts on the CaR by binding to its ECD, taking a similar approach that utilized chimeric receptors and showing that a chimeric receptor comprising the CaR's ECD and mGluR1a's TMDs and COOH tail was activated by Ca_o^{2+}, Mg_o^{2+} and Ba_o^{2+} with EC_{50} values very similar to those of the wild-type CaR. These workers also extended this analysis to define specific residues (e.g., Ser-147 and Ser-170) within the CaR's ECD that may be involved in determining the receptor's apparent affinity for Ca_o^{2+} (41). As described above, these residues are in positions homologous to those of Ser-165 and Thr-188 in mGluR1a. These two serine residues in the CaR and the equivalent residues in mGluR1a and the other mGluRs are thought to play key roles in the binding of these receptors' respective ligands to their ECDs. Moreover, amino acid residues in similar positions within the $GABA_B$ receptors have recently been suggested to play important roles in the binding of GABA. Brauner-Osborne et al. (41) found that mutating Ser-147 to alanine produced a fourfold reduction in the EC_{50} of the CaR for Ca_o^{2+}, whereas a CaR in which Ser-170 was changed to alanine showed no activation by 50 mM Ca_o^{2+}. Therefore, it is possible that the binding of Ca_o^{2+} by the CaR involves residues within the ECD that are equivalent to those within the mGluRs and $GABA_B$ receptors that bind glutamate and GABA, respectively.

It should be noted that there are currently no assays of the binding to the CaR of its physiological ligands. Therefore, studies on the determinants within the ECD that are potentially involved in binding these ligands (41, 317) have so far relied on indirect measures of binding, namely, high Ca_o^{2+}- and other agonist-evoked increases in Ca_i^{2+} or PLC activity (e.g., as assessed by accumulation of inositol phosphates). Clearly mutating a residue within a GPCR need not modify that receptor's function solely through a direct action (e.g., by interfering with the binding of a ligand to a specific amino acid residue). By altering the receptor's conformation, mutating such a residue could also modulate the protein's function indirectly by secondarily perturbing agonist binding and/or subsequent steps involved in activating intracellular signal transduction [e.g., coupling of the receptor to its respective G protein(s)]. Thus further direct structural studies (e.g., using X-ray crystallography) will be necessary to establish definitively whether the serines at amino acid

positions 147 and 170 within the CaR's ECD participate directly or indirectly in Ca_o^{2+} sensing. Moreover, the CaR exhibits a Hill coefficient for its activation by high Ca_o^{2+} of ~3 (15), while the Hill coefficients for activation of the mGluRs by glutamate or by Ca_o^{2+} are ~1 (256). Therefore, it is likely that there are several (probably at least 3 as estimated from the Hill coefficient) binding sites for Ca_o^{2+} within the CaR's ECD and/or elsewhere on the receptor. As discussed in section VI*D*, the fact that the CaR exists on the cell surface principally as a disulfide-linked dimer may contribute to this receptor's apparent positive cooperativity in its binding of Ca_o^{2+}.

It is noteworthy that the agonists of the family C receptors, namely calcium ions, glutamate, and GABA, are small molecules (or ions) that bind predominantly, if not entirely, to these receptors' large ECDs. The other major class of GPCRs that bind their agonists to a substantial extent within their ECDs are the receptors for the glycoprotein hormones: thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, and human chorionic gonadotropin, all of which, in contrast to the agonists for the family C GPCRs, are relatively large heterodimeric glycoproteins (440). In the great majority of the other GPCRs, even those whose agonists are small molecules, such as the biogenic amines, the receptors' agonists have binding sites that likely involve amino acid residues near the extracellular ends of the TMDs, deeper within the TMDs, or in the extracellular loops (454).

D. Role of Cysteines in Receptor Dimerization

The CaR shares with the mGluRs the same relative positions of a total of 20 cysteines: 17 within the ECD, 1 each in the first and second predicted extracellular loops, and 1 in the fifth TMD (58, 157). Clearly these cysteines could be involved in intra- and/or intermolecular disulfide bonds that are important in stabilizing the CaR's tertiary and quaternary structures, e.g., by participating in receptor dimerization. It is of interest in this regard that mGluR5 is a dimer held together by intermolecular disulfide bond(s) present within the first 17 kDa of its NH_2 terminus (389). In this region, the CaR harbors six cysteines, although two of them are within the predicted signal peptide and are likely removed during biosynthesis. Recent studies using site-directed mutagenesis have documented that Cys-129 and Cys-131 are necessary for dimer formation (377), since the CaR migrates on PAGE principally as a monomer when these two cysteines are replaced by serines. Interestingly, the resultant CaR is substantially more sensitive to Ca_o^{2+} than the wild-type receptor, suggesting that these intermolecular disulfide bonds could participate in constraining the receptor in its inactive conformation(s). Although Ray et al. (377) interpreted these findings as showing that the CaR lacking Cys-129 and Cys-131 resides on the cell surface as monomers, we have recently found using immunoprecipitation and cell surface cross-linking that noncovalently bound dimers represent the major form of the cell surface CaR in HEK293 cells transiently transfected HEK293 with a receptor lacking Cys-129 and Cys-131 (M. Bai and E. M. Brown, unpublished data).

E. Functional Importance of Receptor Dimerization

As noted above, the cell surface form of the CaR, at least in transiently transfected HEK293 cells, is principally a disulfide-linked dimer. Recent studies have addressed the question of whether dimerization of the CaR is of functional significance; that is, do the two monomeric partners within these dimers bind calcium and/or other CaR ligands and subsequently activate G proteins and intracellular signaling pathways in a largely independent manner or do the monomers interact functionally in some way? Bai et al. (17) showed that individually inactive CaRs containing either inactivating point mutations within their ECDs or sufficient truncation of their COOH tails to abrogate biological activity showed substantial reconstitution of biological activity when cotransfected in HEK293 cells (Fig. 3) (17). Much less reconstitution of activity, in contrast, occurred when two inactive or partially active CaRs were cotransfected that both harbored ECD mutations, or both had defects (e.g., either truncation of the COOH tail or an inactivating point mutation within the third cytoplasmic loop) within domains of the receptor likely involved in intracellular signaling (17). This result suggested that the CaR has at least two functional domains, i.e., the ECD and the remainder of the receptor. These two domains can complement one another in cotransfections involving mutant receptors having defects in different domains. It is also likely that intermolecular interactions between CaR monomers within the dimeric receptor may contribute to the dominant negative action of some CaR mutations identified in FHH, particularly those exhibiting relatively robust levels of cell surface expression and forming substantial quantities of heterodimers of the mutant and wild-type receptors (14, 15). Such dominant negative CaRs have provided a useful tool for determining the CaR's mediatory role in Ca_o^{2+}-regulated cellular processes (see sect. VIII) (291).

F. Regulation of the CaR by PKC

Activators of PKC, such as phorbol 12-myristate 13-acetate (PMA), substantially reduce Ca_o^{2+}-evoked increases in inositol phosphates and Ca_i^{2+} in bovine parathyroid cells (243, 293, 326, 369, 408). The presence of predicted PKC phosphorylation sites in the CaR's intracellular domains suggests that PKC could participate in modulating the receptor's function by phosphorylating

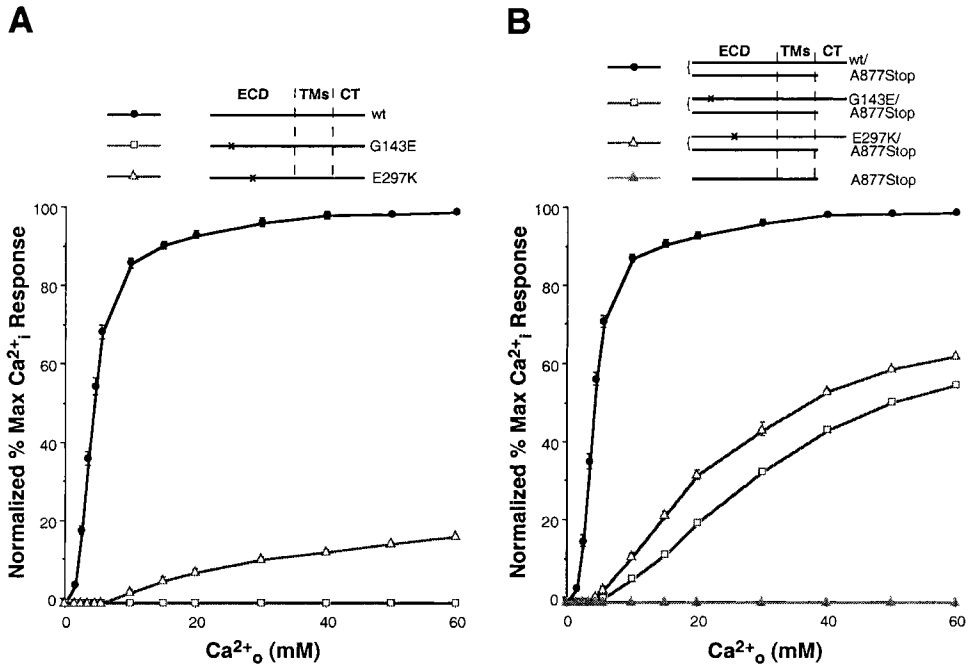

FIG. 3. Cotransfection of inactive mutant CaRs reconstitutes CaR-mediated, extracellular Ca^{2+} (Ca_o^{2+})-elicited cytosolic Ca^{2+} (Ca_i^{2+}) signaling in HEK cells. Responses are normalized to the maximal cumulative Ca_i^{2+} responses observed with cells transfected with normal (wt) receptor alone for both *A* and *B*. *A*: HEK cells were transfected with either wt or one of the two mutant CaRs, G143E or E297K, either of which had very little activity by itself. *B*: cells were transfected with the truncation mutant A877Stop or were cotransfected with A877Stop and the full-length wt (wt/A877Stop) or a mutant CaR, either G143E (G143E/A877Stop) or E297K (E297K/A877Stop). Points are the mean values ± SE (n = 3–9). ECD, extracellular domain of a GPCR; TMs, transmembrane domains. [From Bai et al. (17). Copyright 1999 National Academy of Sciences, USA.]

one or more of these sites (88). Bai et al. (18) recently examined the functional importance of the predicted PKC phosphorylation sites within the human CaR's intracellular domains. The human receptor contains a total of five predicted PKC sites, one each within the second and third ICLs and three within the COOH tail (18, 157). Deleting the two PKC sites within the ICLs had little or no effect on PMA-induced modulation of high Ca_o^{2+}-elicited increases in Ca_i^{2+} in HEK cells transiently transfected with the mutant CaRs. Deletion of the PKC site at residue 888 within the CaR's COOH tail, in contrast, substantially reduced the effect of PMA. Individually removing the two other PKC sites within the tail had relatively little impact on the effect of PMA on the CaR's function, but when all three PKC sites within the COOH tail were deleted, there was a modest further reduction in the effect of PKC activators on high Ca_o^{2+}-evoked Ca_i^{2+} responses (18). Thus the phosphorylation of the CaR's PKC sites, particularly that present at residue 888 in its COOH tail, can account for much of the inhibitory effect of PKC activators on CaR-mediated signaling through the PLC-inositol trisphosphate pathway. The small (~30%) residual effect of PMA that remains after deletion of all of the CaR's PKC sites suggests that PKC can phosphorylate other sites on the CaR and/or regulate other components within this pathway [e.g., G protein(s) and/or PLC].

G. Functional Significance of the CaR's COOH Terminus

Like several other members of the family C GPCRs, the CaR has a long COOH tail, 222 amino acid residues in the case of the bovine CaR (58) and 216 for the human receptor (157). Most of the COOH tail is not needed for the receptor's biological activity. Ray et al. (376) found that CaRs with truncations at amino acid residues 888 and 903 in the COOH tail (the wild-type receptor has 1,078 residues) exhibited biological activities equivalent to that of the wild-type CaR, while those truncated at positions 865 and 874 were inactive, despite exhibiting only ~25% reductions in cell-surface expression (376). Furthermore, mutant receptors with a full-length COOH tail, but with individual residues between positions 874 and 888 replaced with alanines, had relatively normal levels of cell surface expression but markedly reduced biological activities (376), further implicating this region as containing crucial structural determinants required for normal biological activity.

Bai et al. (16) found similar results in their studies of several additional tail-truncated CaRs, albeit with at least one significant difference. Similar to the results of Ray et al. (376), they (16) found that truncation of the CaR's COOH tail beyond residue 870 (e.g., Lys863stop) produced an inactive receptor (16). A mutant CaR truncated at position 877, Ala877stop, was also inactive (16), not unlike the findings of Ray et al. (376) that a CaR truncated at position 874 had no activity. Thus the minimum number of residues in the human CaR required for biological activity is somewhere between 877 and 888, corresponding to a predicted COOH tail of 15–26 residues in length. Furthermore, Bai et al. (16) showed that the mutant receptor, Ser892stop, had an increased level of cell surface expression compared with the wild-type CaR and had a significantly left-shifted EC_{50} for its activation by Ca_o^{2+}

relative to the latter (16). The tail-truncated CaR, Ala877stop, likewise exhibited an increased level of cell surface expression (16, 17), despite its lack of biological activity. These results suggested that there are structural elements within regions of the receptor's COOH tail distal to residue 892 that reduce its cell surface expression in some manner. Ray et al. (376), in contrast, observed neither increased levels of expression nor left-shifted EC_{50} values of their tail-truncated CaRs. We have recently identified, however, a family with autosomal dominant hypocalcemia caused by a large internal deletion within the CaR's COOH tail, producing a loss of most of the normal tail, beginning at amino acid position 895 and ending with the three residues normally present at the receptor's extreme COOH terminus (265). This mutant receptor is expressed at increased levels when transiently transfected in HEK293 cells and shows a left-shifted EC_{50}. Thus this experiment-in-nature provides strong additional support for the hypothesis that truncation within a critical region of the CaR's COOH tail between residues 895 and 1075 can produce an "activated" receptor in vivo.

A recent study has also implicated the CaR's COOH tail in contributing to the positive cooperativity that is characteristic of this receptor (152) as well as in influencing the rate at which the CaR desensitizes after repeated exposures to its agonists. Desensitization refers to a progressive reduction in agonist-mediated activation of a receptor following multiple exposures to that agonist. Given its crucial role as the body's "thermostat" or "calciostat" for Ca_o^{2+}, it is probably not surprising that the wild-type CaR desensitizes little, if at all, when it is exposed to its agonists several times in succession or for extended periods of time (58, 157) (for review, see Ref. 52). This property of the receptor may be crucial in ensuring that the parathyroid gland, for instance, is capable of responding to increases or decreases in Ca_o^{2+} from its normal level in the blood with immediate, CaR-mediated inhibition or stimulation of PTH secretion, respectively. This persistent responsiveness of the CaR in the parathyroid to changes in Ca_o^{2+} occurs despite the fact that ambient levels of Ca_o^{2+} probably produce some degree of receptor activation at all times. In contrast, most other GPCRs show prominent and, in some cases, nearly complete loss of activation following several exposures to their respective agonists (67).

Gama and Breitwieser (152) produced a series of truncated CaRs that had enhanced green fluorescent protein (EGFP) fused to the receptor's COOH terminus. CaRs with truncation and fusion at residues 1024, 908, or 886 were essentially indistinguishable from the wild-type receptor fused to EGFP in their apparent affinities for Ca_o^{2+}, degrees of cooperativity and rates of desensitization (152). A CaR construct truncated and fused at position 868, in contrast, had a reduction in apparent affinity for Ca_o^{2+} and exhibited both reduced cooperativity and accelerated desensitization. The decreased affinity and cooperativity of the latter mutant CaR were mimicked by a point mutation within the COOH tail of the full-length CaR (Thr876Asp), although the latter did not show accelerated desensitization (152). It is not clear whether the presence of the EGFP at the COOH terminus of the CaR truncated at residue 868 conferred some biological activity on this mutant receptor, despite the lack of activity of CaRs truncated to residues 877 (16), 874, or beyond in studies carried out previously by other workers (376). Nevertheless, it seems apparent that the CaR's COOH tail possesses determinants that impact on several important properties of this receptor, namely, its level of cell surface expression, capacity to activate intracellular signaling, degree of positive cooperativity, and/or rate of desensitization.

VII. G PROTEINS AND SIGNAL TRANSDUCTION PATHWAYS TO WHICH THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR COUPLES

A. G Proteins Coupled to the CaR

Most cells express a variety of G proteins that couple diverse GPCRs to their respective downstream effectors. The parathyroid chief cell is no exception, expressing the pertussis toxin-sensitive G proteins, G_{i-1}, G_{i-2} and G_{i-3}, and G_o, as well as the pertussis toxin-insensitive G proteins, G_s, G_q, G_{11}, G_{12}, and G_z (439). The presence of a G protein does not, of course, indicate that it couples to a particular GPCR, and there is relatively little information available defining the G proteins that are directly activated by the CaR. In one of the few studies examining putatively CaR-mediated changes in the activities of various G proteins, Raymond and co-workers (10) showed that the Madin-Darby canine kidney (MDCK) cell line expresses the CaR as assessed by RT-PCR and sequencing using nested CaR primers. Addition of the CaR agonist extracellular Gd^{3+} increased the binding of the photoaffinity-labeled GTP analog [α-^{32}P]GTP azidoanalide (AA-GTP), to $G_{q,11}$, G_{i-2}, and G_{i-3} but not to G_{i-1} in cell membranes prepared from MDCK cells, suggesting that the CaR might couple to and activate these three G proteins. We have observed, however, very low levels of CaR protein expression in MDCK cells as assessed by immunocytochemistry and Western analysis (N. Chattopadhyay, O. Kifor, and E. M. Brown, unpublished data). Thus additional studies using similar approaches will be important in other cell types expressing the CaR at higher levels in which it has been definitively established to modulate cell function in a G protein-dependent manner.

Only indirect studies are available addressing the roles of various G proteins in cells with high levels of CaR

expression. In bovine parathyroid cells, CaR-mediated inhibition of agonist-stimulated cAMP accumulation is abrogated by pretreatment with pertussis toxin (95), suggesting that the CaR inhibits adenylate cyclase via one of the isoforms of G_i expressed in this cell type (see above). One earlier study (141), but not another (56), showed that pertussis toxin blocks the inhibition of PTH secretion mediated by high Ca_o^{2+} or other CaR agonists. Chang et al. (81) have recently shown that CaR agonists also inhibit cAMP accumulation in HEK293 cells stably transfected with the human CaR. The effect of pertussis toxin on this parameter, however, was not examined in this study. Therefore, it is not currently known whether the inhibition of adenylate cyclase in CaR-transfected HEK293 cells occurs through a mechanism involving G_i as opposed to, for example, one entailing release of $G\beta\gamma$ subunits from a pertussis toxin-insensitive, CaR-activated G protein (e.g., G_q) or through an associated, PLC-mediated increase in Ca_o^{2+}, which then inhibits, respectively, $G\beta\gamma$- or Ca^{2+}-inhibited isoforms of adenylate cyclase (for review, see Ref. 423).

The activation of PI-specific PLC by the CaR in bovine parathyroid cells (177) and in CaR-transfected HEK293 cells is pertussis toxin insensitive and most likely occurs through a mechanism involving $G_{q/11}$. In contrast, CaR-mediated stimulation of PI-PLC in ACTH-secreting AtT-20 cells (131) as well as in *X. laevis* oocytes injected with mRNA encoding the CaR (58) is substantially inhibited by pretreatment with pertussis toxin. Therefore, the CaR can apparently utilize distinct signaling pathways, involving either pertussis toxin-sensitive or -insensitive G proteins, to activate PI-PLC depending on the cellular context in which it is expressed. A similar phenomenon has been documented with other GPCRs (302).

B. Intracellular Signaling Systems Regulated by the CaR

1. Phospholipases

The CaR stably expressed in HEK293 cells activates phospholipases C, A_2, and D, whereas CaR agonists have no effects on these phospholipases in nontransfected HEK cells (244). Moreover, CaR agonists stimulate the same three phospholipases in bovine parathyroid cells, presumably acting via the CaR, since high Ca_o^{2+} no longer exerts these effects in parathyroid cells in primary culture, in which CaR expression decreases dramatically after 3–4 days (244). CaR-mediated activation of PI-PLC in parathyroid and CaR-transfected HEK cells appears to entail a direct, G protein-mediated process, probably involving $G_{q/11}$, since this effect is not blocked by pertussis toxin, as noted above. CaR agonist-evoked activation of PI-PLC and attendant mobilization of intracellular calcium have also been documented in several other cell types (19, 40, 63, 79, 151, 290, 291) and appear to be an important mechanism(s) through which the CaR exerts its biological actions.

Activation of PLA_2 and PLD by high Ca_o^{2+} and other CaR agonists are probably indirect, utilizing CaR-mediated, PLC-dependent stimulation of PKC (244), because downregulation or inhibition of PKC largely abolishes these effects. We recently showed that the isoform of PLA_2 activated by the CaR is the cytosolic form of the enzyme and that the mechanism underlying its activation involves phosphorylation of the enzyme by mitogen-activated protein kinase (MAPK) (245a).

In addition to activating PI-PLC, PLA_2, and PLD, recent studies have suggested that the CaR may stimulate the activity of phosphatidylcholine (PC)-specific PLC in calcitonin- and serotonin-secreting sheep parafollicular cells. McGehee et al. (288) showed that inhibitors of PC-PLC partially (e.g., by 50%) blocked the high Ca_o^{2+}-evoked, presumably CaR-mediated stimulation of serotonin secretion in this experimental model (288). Additional studies are needed to document directly activation of this enzyme by the CaR in this and other cells and to elucidate the mechanism through which this effect takes place.

2. MAPKs and tyrosine kinases

Activation of the CaR by its agonists or the calcimimetic CaR activator R-568 [which binds to the TMDs of the receptor and activates it through an allosteric mechanism (320); see sect. XII], stimulates the proliferation of several cell types (276, 290, 291, 463) (see sect. VIII*C*). Many GPCRs are known to stimulate the proliferation of numerous cell types through activation of MAPK signaling cascades (169, 438). In mammalian cells, MAPK have been classified into at least five subfamilies: the extracellular signal-regulated kinases (ERK) MAPK group, the c-Jun NH_2-terminal kinase/stress-activated protein kinases (JNK/SAPK), p38 MAPK and BMK1/ERK5, on the basis of sequence homology and functional properties (159). Of these, the ERK/MAPK subfamily is commonly linked to the control of cellular proliferation.

Tyrosine kinases play important roles in GPCR-mediated stimulation of the ERK/MAPK pathway that occurs through activation of the low-molecular-weight GTP-binding protein, p21 Ras (112, 250). Recent studies have implicated three distinct classes of tyrosine kinases as key mediators of GPCR-induced Ras-activation. These are *1*) transactivated receptor tyrosine kinases, such as the epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) receptors; *2*) integrin-based focal adhesion kinases (FAK), which are activated following dimerization of integrins induced by binding of their extracellular domains to their respective ligands in the extracellular matrix; and *3*) Src family kinases (274), which are nonreceptor tyrosine kinases resident within the cytoplasm.

There is a subsequent recruitment upon the "scaffolds" provided by transactivated receptor tyrosine kinases and/or focal adhesion complexes of the adapter proteins [e.g., Shc and Gab1 (78, 437)], signaling proteins (like Grb2) and guanine nucleotide exchange factors (i.e., SOS1) required for activating Ras (121, 169, 438). In some cases, GPCRs can utilize a Ras-independent mechanism for activation of MAPK that is thought to be mediated by PKC (176, 262).

Biochemical pathways involving MAPK that are similar to those defined for various other GPCRs may underlie the CaR-mediated stimulation of cellular proliferation in some cell types. McNeil et al. (291) employed wild-type rat-1 fibroblasts as well as those stably transfected with a naturally occurring, dominant negative mutant of the CaR [e.g., Arg795Trp (14, 15)] to show that CaR activation substantially increases c-Src tyrosine kinase activity in wild-type rat-1 fibroblasts but not in those stably transfected with the dominant-negative CaR construct. Activation of the receptor-induced tyrosine phosphorylation of p125 FAK, a focal adhesion kinase, as well as unidentified proteins of 63–65 kDa. CaR agonists likewise produced associated 10- to 25-fold increases in Erk-1 kinase activity, one of the MAPK family members, that was completely blocked by the dominant negative CaR construct. Herbimycin, a tyrosine kinase inhibitor with selectivity for Src, blocked the increases in both Erk-1 kinase activity and thymidine incorporation, indicating that CaR-mediated activation of Src tyrosine kinase activity took place upstream of these latter two biological responses. Furthermore, PD98069, a specific inhibitor of MAPK kinase (MEK1), a protein kinase immediately upstream of MAPK that activates the latter, also inhibited CaR agonist-stimulated thymidine incorporation. These investigators also used EGF as a positive control for a receptor-mediated stimulation of cell proliferation occurring via the MAPK cascade. Interestingly, in cells transfected with the dominant negative CaR construct, stimulation of Erk-1 by EGF was substantially reduced compared with that observed in wild-type rat-1 fibroblasts (291). Thus there may be some type of cross-talk between the CaR and the EGF receptor, similar to the transactivation of receptor tyrosine kinases described for other GPCRs (274).

Further studies are required to understand how the CaR activates the MAPK pathway. Our current understanding of how other GPCRs that couple to signaling pathways similar to those utilized by the CaR to activate MAPK makes it reasonable to address the following issues in future studies: to what extent in different cell types does CaR-stimulated, Ras-dependent MAPK activation involve transactivation of receptor tyrosine kinases versus stimulation of focal adhesion kinases? What are the relative contributions of $G_{q/11}$- and G_i-mediated activation of Ras in different CaR-expressing cells? Is there an additional Ras-independent pathway for activation of MAPK involving PKC? Furthermore, additional studies are needed to determine whether CaR activation results in the stimulation of other MAPK cascades, such as JNK/SAPK or p38.

3. Adenylate cyclase

As noted above, the CaR confers high Ca_o^{2+}-induced inhibition of cAMP accumulation upon parathyroid cells and HEK293 cells stably transfected with the CaR (81, 95, 386). In the case of parathyroid cells, there is likely a direct, G_i-mediated inhibition of adenylate cyclase. A similar, pertussis toxin-sensitive, high Ca_o^{2+}-evoked inhibition of cAMP accumulation that is likely CaR-mediated takes place in tubules isolated from the medullary thick limb (MTAL) of the mouse kidney (426). Recent studies carried out using tubules isolated from the MTAL of the rat kidney, however, have suggested that high Ca_o^{2+}-induced inhibition of agonist-stimulated cAMP accumulation (426) occurs through an indirect, albeit pertussis toxin-sensitive, mechanism involving arachidonic acid (140); that is, addition of arachidonic acid to suspensions of tubules produced a pertussis toxin-sensitive reduction in cAMP accumulation (140). Since the cloned CaR activates PLA_2 in CaR-transfected HEK cells (244), and high Ca_o^{2+}, likely acting through the CaR, stimulates PLA_2 activity in parathyroid cells (36) and cells of the cortical thick ascending limb (CTAL) of the rat kidney (449, 450), pertussis toxin-sensitive, CaR-induced inhibition of cAMP accumulation in some tissues could potentially utilize this indirect mechanism. In bovine parathyroid cells, however, we have found no effect of adding exogenous arachidonic acid on agonist-stimulated cAMP accumulation under conditions where high Ca_o^{2+} inhibits the latter by 80% or more (R. Butters and E. M. Brown, unpublished data).

In some cells expressing the CaR, high Ca_o^{2+} can inhibit cAMP accumulation through a pertussis-toxin-insensitive mechanism involving a CaR-evoked increase in Ca_i^{2+}, which then inhibits a Ca^{2+}-sensitive form of adenylate cyclase (119). Thus it is likely that the CaR can inhibit adenylate cyclase activity and, in turn, cAMP accumulation through several different mechanisms involving not only direct G_i-mediated inhibition of this enzyme but also indirect mechanisms. Interestingly, in AtT-20 cells (131), as well as in cells isolated from pituitary adenomas (390), high Ca_o^{2+} raises cAMP levels. Whether this action involves stimulation of adenylate cyclase [via a Ca^{2+}-stimulated isoform of the enzyme, for example, as opposed to a direct G_s-mediated activation (423)] requires further study.

C. Role of Caveolae in CaR-Mediated Signal Transduction

Kifor et al. (245) have recently shown that the CaR in bovine parathyroid cells resides principally within caveolae-like structures (245). Caveolae are small (~50 nm), flasklike structures within the plasma membrane that are known to participate in potocytosis and transcytosis, processes by which bulk substances within the extracellular fluid compartment are taken up by cells for internal utilization (i.e., by potocytosis = "cell drinking") or taken up, transferred across the cell, and released at the opposite cell surface (e.g., by transcytosis) (8). They are specific microdomains within the plasma membrane that differ from the rest of the cell membrane in their lipid composition, being rich in cholesterol and sphingolipids. They generally comprise 10% or less of the total surface area of most cells. Recently, accumulating evidence suggests that caveolae can serve as key cellular "signaling centers," where GPCRs, G proteins and other important elements in signal transduction, such as various isoforms of PKC and Src family tyrosine kinases, are localized and organized into signaling complexes (8, 258, 268, 403).

Bovine parathyroid cells are relatively rich in caveolin-1 (245), one of a family of integral membrane proteins [e.g., caveolins-1, -2, and -3 (258)] that are thought to contribute importantly to the structure and function of caveolae by participating in their structural organization and through binding to and modulation of important signaling molecules resident within them, such as G proteins (264). About 80% of the CaR within parathyroid cells resides within caveolae-like structures, independent of the state of receptor activation, as assessed by biochemical isolation of these structures using either detergent-based or detergent-free methods that have been widely used for this purpose (245). The bulk of the cellular CaR in bovine parathyroid cells can be immunoprecipitated with anti-caveolin-1 antibodies, further supporting caveolae as a key site of localization of the receptor (245). The physical association of the CaR and caveolin-1 suggests that the receptor either interacts directly with the latter or does so indirectly by interacting with other proteins that bind to both CaR and caveolin-1. Such intermolecular interactions could potentially modulate the receptor's structure and function.

Bovine parathyroid caveolae contain several additional proteins of potential relevance to signaling via the CaR, including $G_{q/11}$ and both PKC-α and PKC-ζ (245). Interestingly, activation of the CaR promotes tyrosine phosphorylation of caveolin-1 in parathyroid cells, although the functional significance of this effect is currently unknown (245). It will be of interest to determine whether the CaR expressed in other cell types is also present in caveolae and to elucidate the importance of this cellular localization in the mechanisms through which this receptor regulates the diverse cellular functions described in section VIII.

VIII. CELLULAR PROCESSES REGULATED BY THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR

A. Overview of Cellular Processes Regulated by the CaR

The CaR regulates numerous cellular processes (Table 1), likely acting via the intracellular signaling pathways just delineated. Most of these are covered later in discussions of the CaR's roles in the various cells expressing it that are either involved (sect. X) or uninvolved (sect. XIII) in systemic Ca_o^{2+} homeostasis. In a few cases, however, where the relevant processes and their control by the CaR are not addressed in other parts of this review, these are described briefly in the present section.

B. Secretion

The roles of the CaR in regulating PTH and CT secretion (288, 402, 481) are well-established, and the feedback control of these hormones by Ca_o^{2+} is crucial elements in the homeostatic mechanisms governing systemic Ca_o^{2+} homeostasis (see sect. X). The CaR also likely represents the cellular mechanism underlying the known stimulatory actions of high Ca_o^{2+} on the secretion of several additional hormones by cells that do not participate in systemic Ca_o^{2+} homeostasis [e.g., of gastrin (374), ACTH (131, 139), growth hormone (390), and insulin (130, 261)]. High Ca_o^{2+} likewise stimulates PTH-related peptide (PTHrP) secretion from several types of cells, including normal keratinocytes (189) and cervical cells (255), squamous cancer cells (295), JEG-3 choriocarcinoma cells (187), and H-500 Leydig cell cancer cells (399). The presence of the CaR in these cells has only been examined in the case of H-500 cells, however, which express readily detectable amounts of CaR mRNA and protein (399).

Parathyroid cells are not the only cell type in which high Ca_o^{2+} inhibits hormonal secretion. Raising Ca_o^{2+} also inhibits the release of glucagon from the pancreatic islets under some circumstances (130, 261). Moreover, high levels of Ca_o^{2+} inhibit the secretion of renin by the juxtaglomerular apparatus (JGA) of the kidney (145). Although the CaR is expressed in multiple regions of the kidney (379, 380, 469), it is not currently known if the CaR is expressed in the JGA and if it mediates the inhibitory action of high Ca_o^{2+} on renin secretion.

TABLE 1. *Processes known or likely to be regulated by the CaR*

Process	Cell Type	Effect	Discussed in Section	Reference No.
Secretion				
PTH	Parathyroid	−	x*A*	61
CT	C cell	+	x*B*	288, 402, 481
ACTH	Pituitary	+	xiii*C*	131, 139
Gastrin	G cells	+	xiii*G*	374
Insulin	Beta cells	+	xiii*H*	236
Glucagon	Alpha cells	−	xiii*H*	130, 261
Growth hormone	Pituitary	+	xiii*C*	390
PTHrP	Multiple	+	xiii*E*	399
Channels/transporters				
NCC	Multiple	+	x*A*, xiii*A*	471–473
VDCC	C cell	+	x*B*	288
CAKC	Multiple	+	xiii, *A* and *B*	90, 92, 441
K^+ channel	Parathyroid	−	x*A*	442
Apical K^+ channel	Kidney	−	x*C*	449, 450
Aquaporin-2	Kidney	−	x*C*	400, 401
Chemotaxis				
	Osteoblasts	+	x*D*	161, 463
	Stromal cell	+	x*D*	462
	Macrophage	+	xiii*D*	421, 464
Proliferation				
	Parathyroid	−	x*A*	196
	Colon crypt cell	−	xiii*F*	68, 70, 114, 225
	Keratinocyte	−	xiii*F*	30, 191, 351
	Fibroblast	+	viii*B*	291
	Ovarian surface cell	+	viii*B*	290
Differentiation				
	Keratinocyte	+	viii*C*, xiii*F*	30, 191, 351, 435
	Goblet cell	+	viii*C*	31, 85, 151
	Mammary cell	+	viii*C*, xiii*E*	97, 289
Apoptosis				
	Fibroblast	−	viii*D*	267
	CaR-transfected HEK	−	viii*D*	267
	Prostate cancer cell	−	viii*D*	267
Gene expression				
PTH*†	Parathyroid	−	x*A*	44, 394, 395, 413, 467
VDR*	Parathyroid	+	x*C*	394
CaR*	Pituitary	+	xiii*C*	131
Calmodulin*	C cell	+	x*B*	425
Calcitonin*	C cell	+	x*B*	425
$CalbindinD_{28K}$*	Kidney	+	x*C*	105
Aquaporin-2†	Kidney	+	x*C*	400
P-cadherin†	Squamous cell cancer	+	xiii*F*	448

* Transcriptional regulation. † Posttranscriptional regulation. The reference no. column is not exhaustive; see listed sections for additional references. PTH, parathyroid hormone; CT, calcitonin; PTHrP, PTH-related peptide; NCC, nonselective cation channel; VDCC, voltage-dependent calcium channel; CAKC, calcium-activated potassium channel; VDR, vitamin D receptor; CaR, extracellular calcium-sensing receptor.

C. Proliferation, Differentiation, and Apoptosis

The CaR is thought to regulate three important cellular processes that are important determinants of cell fate under both normal and pathological conditions: proliferation, differentiation, and apoptosis. Discussions of the probable roles of this receptor in regulating cellular differentiation in epithelial cells of the breast and colon crypts, as well as in keratinocytes, may be found in section xiii, *E–G*, respectively. The CaR also inhibits the proliferation of parathyroid cells (see sect. x*A*) and likely mediates the stimulatory effect of high Ca_o^{2+} on the proliferation of cells of the osteoblastic lineage (see sect. x*D*) as well as the inhibitory actions of high Ca_o^{2+} on the proliferation of breast cells, colon crypt cells, and keratinocytes (see sect. xiii, *E–G*). Finally, the CaR stimulates the proliferation of rat-1 fibroblasts and ovarian surface cells, actions that likely involve CaR-mediated activation of MAPK, as described in section vii*B*.

Apoptosis is a physiological form of cell death that plays critical roles in tissue homeostasis, development, and immune defense by ridding the body of damaged, dead, or unwanted cells. Common features of cells undergoing apoptosis include condensation of chromatin, internucleosomal DNA fragmentation (DNA laddering), cell shrinkage, and activation of the caspase family of proteases. Apoptosis has recently been reviewed extensively (190, 210, 217, 321). A role for the CaR in protecting

against apoptosis was found using AT-3 rat prostate carcinoma cells, c-*myc*-overexpressing rat 1A fibroblasts, and CaR-transfected HEK cells (267). Both AT-3 cells and rat 1A fibroblasts express the CaR as assessed by immunocytochemistry and Northern analysis (267). Furthermore, raising the level of Ca_o^{2+} to 5–10 mM reduced by 50% or more the extent of apoptosis in AT-3 cells or rat 1A fibroblasts that was induced by infection with Sindbis virus or c-*myc* overexpression/serum deprivation, respectively, as assessed by determining DNA laddering and quantitating cell viability (267). As additional evidence that this action was mediated by the CaR, raising the level of Ca_o^{2+} substantially reduced the extent of apoptosis induced by Sindbis virus in CaR-transfected HEK293 cells but not in nontransfected HEK293 cells (267). Furthermore, elevating Ca_o^{2+} had no effect on viral replication in either cell type. Taken together, these findings suggested that the activated CaR protects these three cell types from apoptosis induced by various agents.

Of interest, the structurally and functionally related mGluRs can protect neurons against apoptosis induced by either oxygen-glucose deprivation (65) or addition of the neurotoxic β-amyloid peptides (111). In addition, 5-oxo-eicosatetraenoic acid, which interacts with a specific GPCR on its target cells (328, 355), blocks apoptosis triggered by inhibition of 5-lipoxygenase in human prostate cancer cells (164a). Thus a protective effect against apoptosis may be shared by many GPCRs, potentially by stimulating Ras, which then exerts an antiapoptotic effect either by stimulating PKB/Akt via PI 3-kinase or through activation of NFκB (128). However, it is currently unknown whether the antiapoptotic effects of activating the CaR result from activation of these pathways.

D. Gene Expression

High Ca_o^{2+} modulates the expression of several genes inhibiting the PTH gene in the parathyroid gland (44, 394, 395, 413, 467) and increasing the expression of calbindin D_{28K} in rat kidney (105) and VDR gene in the chick parathyroid (394). More recently, Suzuki et al. (425) have shown that high Ca_o^{2+} increases the promoter activities and mRNA levels of the CaR, calmodulin, and calcitonin genes in C cells, while Emanuel et al. (131) have demonstrated a high Ca_o^{2+}-induced increase in the level of the mRNA for the CaR in AtT-20 cells. In some cases, vitamin D and Ca_o^{2+} exert synergistic effects in inhibiting [e.g., the PTH gene in the parathyroid gland (394, 467)] or increasing gene expression [viz., of calbindin D_{28K} in rat kidney (105) or the VDR in chick parathyroid (394)]. Because the CaR is expressed in all of the cell types noted above, it is a strong candidate for mediating these various actions of Ca_o^{2+} on gene expression, although its mediatory role has not been formally proven to date.

There are at least two putative molecular mechanisms that underlie these high Ca_o^{2+}-elicited, presumably CaR-mediated actions on gene expression. *1*) In C cells and probably in several other cell types, the TTF-1 transcription factor is activated by the CaR through a mechanism that likely involves an increase in Ca_i^{2+} (425). *2*) In parathyroid cells and other cells in which gene transcription is regulated by Ca_o^{2+}, it has been suggested that the redox factor, ref-1, serves as a transcription factor that negatively regulates the PTH gene (331, 332). Further studies are needed, however, utilizing the cloned CaR to document directly that the effect of this protein on PTH gene transcription is mediated by the CaR and to investigate the underlying mechanism(s) of action.

In addition to regulating the levels of the mRNAs for various genes, high Ca_o^{2+} and the CaR can also likely modulate the activities of proteins in a posttranscriptional or posttranslational manner. For instance, induction of hypercalcemia in rats reduces the level of the aquaporin-2 water channel protein, without altering the expression of its RNA (400). In addition, in a CaR-expressing squamous cancer cell line, elevating Ca_o^{2+} stimulates the translocation of the adhesion molecule P-cadherin to the plasma membrane and increases the total amount of cellular P-cadherin protein without affecting its mRNA level (448). The latter effect was blocked by an inhibitor of tyrosine kinase. Therefore, although only limited data are available related to the possible actions of the CaR on the expression of the mRNAs and/or proteins for various genes, including the CaR gene itself, available data suggest that it exerts several such actions, in addition to its well-known effects on short-term parameters of cellular function, such as modulating secretion, ion channel activity, and ion transport.

IX. OTHER POTENTIAL EXTRACELLULAR CALCIUM-SENSING RECEPTOR AGONISTS AND MODULATORS AND THE ROLE OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR AS AN INTEGRATOR OF DIVERSE PHYSIOLOGICAL SIGNALS

A. Mg_o^{2+}

The effects of elevated levels of Mg_o^{2+} have long been known to mimic those of Ca_o^{2+} in certain cell types. For instance, raising Mg_o^{2+} inhibits PTH secretion from parathyroid cells (60, 171), reduces agonist-stimulated cAMP accumulation in the parathyroid (50) and kidney (284), and diminishes renal tubular reabsorption of Mg^{2+} (as well as Ca^{2+}) (358, 359, 361). The cloning of the CaR made it possible to demonstrate directly that not only Ca_o^{2+} but also Mg_o^{2+} can serve as a CaR agonist (58, 71, 81, 126, 393). Generally, as observed in bovine parathyroid cells, Mg_o^{2+}

is about two- to threefold less potent than Ca_o^{2+} as a CaR agonist in activating PLC (81), producing transient increases in Ca_i^{2+} (71), stimulating the activity of PLA_2 (393), and inhibiting cAMP accumulation (81). In addition, Mg_o^{2+} acts as a partial agonist for many of these actions, producing maximal effects that are less than those elicited by Ca_o^{2+} (393).

It is not currently clear whether the differences between the actions of Ca_o^{2+} and Mg_o^{2+} on the CaR are entirely accounted for by intrinsic differences in their capacities to interact with the receptor and/or other contributory factors. For instance, in the absence of a reliable binding assay for assessing how various agonists interact with the CaR, it has been necessary to infer the interaction of these agents with the receptor indirectly, through changes in downstream biological responses (e.g., various phospholipases and/or adenylate cyclase). Moreover, Mg_o^{2+} could exert additional actions on CaR-expressing cells that interfere with its CaR-mediated actions, such as blocking Ca^{2+} influx pathways, thereby reducing the sustained phase of PLC activation that is dependent on uptake of Ca_o^{2+}. The latter actions could potentially account, at least in part, for some currently unexplained differences in the potencies of Ca_o^{2+} and Mg_o^{2+} in eliciting CaR-mediated biological responses in various cells. For instance, raising Mg_o^{2+} clearly exerts Ca_o^{2+}-like actions on the parathyroid in vitro (60, 171, 430) and in vivo (432), but it has little, if any, effect on calcitonin secretion in vitro in some systems (288), perhaps because Mg_o^{2+} blocks key Ca^{2+}-permeable channels.

Because serum levels of Mg_o^{2+} are, if anything, slightly lower than those for Ca_o^{2+}, can Mg_o^{2+} serve as a physiologically relevant CaR agonist in vivo? Genetic diseases of the CaR suggest that it does, in fact, function as a Mg_o^{2+} sensor in vivo (49, 420); that is, individuals with disorders with inactivating mutations of the CaR tend to have high-normal or mildly elevated levels of Mg_o^{2+} (281), whereas those with activating mutations can have mild hypomagnesemia (344). Indeed, there is a positive correlation between serum Mg^{2+} and Ca^{2+} in persons with FHH, such that those families with more severe hypercalcemia tend to have higher levels of serum Mg_o^{2+} (281). In contrast, there is an inverse correlation between serum Mg^{2+} and Ca^{2+} in primary hyperparathyroidism (281), perhaps because hypercalcemia tends to promote urinary magnesium excretion (358). A particularly striking example of an individual with "resetting" of both Ca_o^{2+} and Mg_o^{2+} was the homozygous offspring of related parents with an inactivating mutation of the CaR who was only identified serendipitously at the age of 35 years (2). Both serum Mg_o^{2+} and Ca_o^{2+} were elevated to levels ~50% above normal, in association with a concentration of serum PTH that was at the upper limit of normal and normal renal function (2).

There may be several ways in which Mg_o^{2+} acts via the CaR to control its own homeostasis in vivo. All of the polycationic agonists (e.g., di- and trivalent cations and polycations such as neomycin) potentiate one another's actions on the CaR (60, 393). Therefore, an increase in Mg_o^{2+} may activate the CaR by, in effect, sensitizing it to Ca_o^{2+}. Furthermore, there may be specific microenvironments where Mg_o^{2+} differs from its level in blood. A good example is the level of Mg_o^{2+} within the renal tubule. Because proximal tubular reabsorption of Mg^{2+} is less than that for Na^+, Cl^-, Ca^{2+}, and water, the level of Mg_o^{2+} rises progressively along the nephron and is 1.6- to 1.8-fold higher in the thick ascending limb than in the initial glomerular filtrate (123). This increase in Mg_o^{2+} within the tubular fluid is probably sufficient to activate CaRs in the CTAL that are thought to regulate the reabsorption of both Ca^{2+} and Mg^{2+} (181).

In addition to the inhibitory effect of raising Mg_o^{2+} on PTH secretion, lowering Mg_o^{2+} also reduces PTH secretion (6, 7) and, to a lesser extent, the actions of PTH on its target tissues in kidney and bone (216). The effects of low levels of Mg_o^{2+} on the mineral ion homeostatic system, however, do not appear to involve the CaR, and the molecular mechanism(s) underlying them is poorly understood.

B. Spermine

Spermine is an effective CaR agonist at ~100 mM to 1 mM (367). Like di- and trivalent cations, it activates PLC, thereby promoting transient increases in Ca_i^{2+} owing to mobilization of intracellular calcium stores in CaR-transfected HEK293 cells and inhibits PTH release from bovine parathyroid cells (367). As with other polycations, such as the aminoglycoside antibiotics (237), the number of charges on the polyamines is an important determinant of their potency as CaR agonists. Their order of potency (spermine > spermidine > putrescine) reflects the fact that these three polyamines contain 4, 3, and 2 free amino groups, respectively (367). Although the concentrations of spermine needed to activate the CaR are relatively high (~millimolar) in the presence of low levels of Ca_o^{2+} (e.g., 0.5 mM), when Ca_o^{2+} is raised to levels approaching those found in vivo (~1.5 mM), concentrations of spermine in the range of 100 mM modulate the CaR's activity, because of the self-potentiating actions of polycationic CaR agonists (see above). The levels of spermine present in vivo are on the order of 100 mM, and in specific microenvironments, such as in the gastrointestinal tract (where bacteria produce spermine) and in the brain (where spermine can be cosecreted with neurotransmitters), concentrations of spermine in extracellular fluids can approach 1 mM (367). Therefore, it is entirely possible that the CaR serves as a physiologically relevant "receptor" for spermine in vivo under certain circumstances, although this

possibility has not been formally tested. There are a number of other highly cationic substances present in vivo, such as protamine, which could conceivably also act on the CaR in a similar manner (59).

C. Amyloid β-Peptides

Vassilev et al. (470) have shown that amyloid β-peptides (Aβ) can serve as CaR agonists in vitro (470). Micromolar concentrations of Aβ-(1—40) potently activate nonselective cation channels (NCC) in neurons from wild-type mice but not in those from mice with knockout of the CaR, as well as in CaR-transfected but not in nontransfected HEK293 cells (470). These actions could potentially be mediated by fibrillar forms of Aβ, since fibrillar deposits of these peptides in the brains of persons suffering from Alzheimer's disease are stained by anionic dyes, such as Congo red. These dyes have a spacing of negative charges approximating that present in spermine (e.g., a known polycationic CaR agonist) for its positive charges. Moreover, it is possible that Aβ-fibrils deposited in brain regions involved in the pathology of Alzheimer's disease, where the CaR is expressed at substantial levels, such as hippocampus, could modulate the activity of these CaRs, contributing to the pathophysiology of this disorder in ways that are currently not understood.

D. Ionic Strength

Quinn et al. (366) have shown that alterations in ionic strength produce parallel changes in the CaR's EC_{50} for high Ca_o^{2+} and other CaR agonist-evoked increases in Ca_i^{2+} (366); that is, an increase in ionic strength reduces the receptor's sensitivity to elevations in Ca_o^{2+} and vice versa. Similar observations were made independent of the nature of the monovalent cation (e.g., sodium or choline) or anion (chloride or iodide) employed to modify ionic strength. Moreover, changes in osmolality per se (i.e., obtained by substituting sodium chloride with sucrose) had no effect on Ca_o^{2+} sensing by the CaR, indicating that ionic strength was the responsible factor (366). Similar effects were noted with the use of Mg_o^{2+} or spermine as the CaR agonist, indicating that the observed effect of ionic strength was not agonist specific.

Changes in ionic strength sufficient to modify the CaR's EC_{50} to the point where the level of Ca_o^{2+} would be expected to become overtly abnormal are seldom encountered in vivo except in severe illness. Nevertheless, substantial changes in ionic strength can take place in specific microenvironment, even in normal physiological states. For instance, the concentration of sodium chloride (and, therefore, ionic strength) in the urine can vary from ~50 to 300 mM. Therefore, the CaR on the apical membrane of the IMCD could experience alterations in ionic strength more than sufficient to modify substantially its EC_{50} for Ca_o^{2+} and other CaR agonists. Comparable changes in ionic strength might be encountered by CaR-expressing cells exposed to the contents of the gastrointestinal tract, particularly in its more proximal portions (e.g., stomach and upper small intestine). Finally, epithelia that transport electrolytes and water at different rates can likely generate sufficiently large changes in ionic strength to modify the CaR's function. In the thick ascending limb (427) of the loop of Henle of the kidney, for instance, sodium chloride is reabsorbed in excess of water, because the epithelial cells of this portion of the nephron exhibit a very low permeability to water. Therefore, CaRs at the basolateral aspect of these epithelial cells could encounter elevated levels of ionic strength relative to those in the initial filtered urine that might alter their Ca_o^{2+}-sensing capacity. To date, however, there have been no studies that have directly documented the expected alteration of Ca_o^{2+} sensing in vivo in a cell type endogenously expressing the CaR that has been exposed to a physiologically relevant change in ionic strength.

The effects of ionic strength on the sensing by the CaR of its various polycationic agonists raise the possibility of an "electromotive" mechanism of activation of the receptor by these agents (366); that is, polycations might screen charges on the receptor, which then changes the conformation of the ECD or other regions of the receptor from its inactive to its active state (366). Such a mechanism could perhaps explain why such a wide variety of polycations, ranging from divalent and trivalent cations to small organic polycations, e.g., neomycin (55, 383), and even substantially larger polycationic proteins, including protamine (59), can all activate the CaR. Moreover, in general, the greater the total number of its positive charges, the greater the potency of a given molecule as a CaR agonist (preparations of polyarginine with a chain length averaging 100 residues act at nM levels). Nevertheless, there must be some steric and/or other factors in addition to just the total number of charges that contribute to activation of the receptor by polycationic agonists. For example, while the trivalent lanthanides exhibit micromolar potencies as CaR agonists (57), gallium (Ga^{3+}) is only a weak activator of the receptor in parathyroid cells (382) and in CaR-transfected HEK293 (S. Quinn and E. M. Brown, unpublished data). In addition, there are differences in potency among the aminoglycosides that are not explained by differences in the number of charges alone (237).

E. Amino Acids

Conigrave et al. (109) have recently shown that a variety of amino acids can serve as positive modulators of the CaR, potentiating the actions of the receptor's poly-

cationic activators in their presence (e.g., of ≥1 mM Ca_o^{2+}) but not in their absence. This action is stereoselective for most amino acids, with L-amino acids being severalfold more potent than D-amino acids. Although individual amino acids exhibit relatively low potencies for activating the CaR, doing so in the millimolar range, a mixture of amino acids emulating that present in fasting persons has a substantial impact on the receptor's sensitivity to its polycationic agonists, decreasing the EC_{50} for Ca_o^{2+} by 20–40% (109). Furthermore, the order of potency for the effects of amino acids on the CaR, with aromatic amino acids being most potent, is highly reminiscent of that for the known effects of various amino acids in stimulating the secretion of gastrin or gastric acid (109). It is possible, therefore, that the CaR mediates these latter actions and functions in vivo more generally as a "nutrient receptor" than as a sensor responding solely to mineral ions (and other polycations).

Indeed, it is quite possible that the CaR mediates known effects of changes in protein intake on mineral ion metabolism. For instance, high protein intake substantially increases urinary calcium excretion (69), whereas low protein intake has recently been shown to produce nearly a doubling of serum PTH levels in normal young women (241). Thus the recognition that the CaR responds to amino acids as well as to mineral ions may provide evidence for a fundamental role of this receptor in integrating protein and mineral ion metabolism (109).

F. The CaR as an Integrator of Diverse Physiological Signals

The foregoing discussion emphasizes that the CaR is capable of responding to several different agonists or modulators that are present in vivo and whose concentrations are or could potentially be within a range capable of modulating the receptor's activity, including Ca_o^{2+}, Mg_o^{2+}, spermine, Aβ, changes in ionic strength, and amino acids. It is an oversimplification, therefore, to think of the CaR as simply a Ca_o^{2+} sensor. Depending on the particular microenvironment within which it resides, the CaR may be exposed to sufficiently high levels of CaR agonists and/or modulators other than Ca_o^{2+} to substantially modify its activity. Because polycationic CaR agonists potentiate one another's actions on the receptor, a relatively small change in the concentration of one agonist can markedly change the apparent affinity of the receptor for another (60, 367, 393). Much additional work is required, however, to characterize various microenvironments in terms of their content(s) of CaR agonists or modulators other than Ca_o^{2+} to assess more fully their contributions to CaR-mediated biological responses. Nevertheless, it is possible that the CaR integrates multiple physiological signals, thereby enabling it to respond in an appropriate manner to the requirements of a specific tissue or microenvironment (see also sects. X, XI, and XIII).

X. THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR'S CELLULAR DISTRIBUTION AND FUNCTIONS IN TISSUES INVOLVED IN MINERAL ION HOMEOSTASIS

A. Parathyroid

The parathyroid glands of humans (162, 246), rats (13), mice (196), rabbits (71), and chickens (126) express abundant CaR mRNA and protein, as assessed using Northern analysis and immunohistochemistry and Western analysis, respectively. Recent studies of inherited diseases of Ca_o^{2+} homeostasis caused by inactivating mutations in the CaR gene and of mice with targeted disruption of this gene strongly support the receptor's central role in mediating Ca_o^{2+}-regulated PTH secretion. Humans heterozygous for inactivating mutations as well as mice heterozygous for knockout of the CaR show modest (10–20%) rightward shifts in the "set point" of the parathyroid gland for Ca_o^{2+} (the level of Ca_o^{2+} half-maximally inhibiting PTH secretion). In humans homozygous for inactivating CaR mutations, who suffer from NSHPT, as well as in mice homozygous for knockout of the receptor, there is much more severe "resistance" of the parathyroid glands to Ca_o^{2+}. In both instances, there is severe hypercalcemia (with elevations in Ca_o^{2+} that are 50% or more above the upper limit of normal) as well as markedly elevated levels of serum PTH. Furthermore, persons harboring activating mutations in the CaR, which render the parathyroid cell overly sensitive to the suppressive action of elevated Ca_o^{2+} on PTH secretion, exhibit hypocalcemia accompanied by inappropriately normal or even low levels of serum PTH (that is, a stimulus which normally increases PTH secretion fails to do so). Thus these experiments-in-nature afford compelling evidence for the central, nonredundant role of the CaR as a mediator of high Ca_o^{2+}-inhibited PTH release.

The intracellular mechanism(s) through which the CaR inhibits PTH secretion, however, remains an important unresolved issue. As outlined above, the CaR modulates diverse intracellular signaling pathways, activating PLC, PLA_2, and PLD as well as the MAPK pathway and inhibiting adenylate cyclase (see sect. VII). Products of the stimulation of PLC (i.e., inositol trisphosphate, which causes a transient increase in Ca_i^{2+}, and/or diacylglycerol), PLA_2 (e.g., yielding arachidonic acid and/or the products of its further metabolism), or PLD (i.e., generating phosphatidic acid), the high Ca_o^{2+}-elicited, sustained increase in Ca_i^{2+} or decrease in cellular cAMP and/or other factors have all been suggested as central mediators of high Ca_o^{2+}-inhibited PTH secretion (for review, see Ref.

52). In virtually all instances, however, high Ca_o^{2+}-evoked alterations in the relevant mediators can be dissociated from concomitant changes in PTH secretion under appropriate conditions. Indeed, even the crucial step(s) along the pathway for Ca_o^{2+}-regulated PTH secretion that is controlled by the CaR remain largely unknown (e.g., from the budding of secretory granules at the Golgi apparatus to their final exocytosis at the cell surface). Thus, despite rapid recent advances in the elucidation of a number of the molecular components of the exocytotic apparatus in other cell types and the cloning of the CaR, much remains to be learned about how the parathyroid cell responds via the CaR to Ca_o^{2+} in a fashion directly opposite to that exhibited by most other secretory cells.

The CaR likely also exerts a tonic suppressive action on parathyroid cellular proliferation, since humans with NSHPT who are homozygous for inactivating mutations of the CaR (49) and mice homozygous for knockout of this receptor (196) exhibit marked parathyroid cellular hyperplasia (in contrast, no such mutations in the CaR gene have been found in pathological parathyroid glands from patients with primary or secondary hyperparathyroidism). Furthermore, treating rats with renal impairment owing to subtotal nephrectomy with the calcimimetic CaR activator R-568 prevents the parathyroid hyperplasia that otherwise develops in the setting of renal insufficiency (445). Although the receptor may inhibit parathyroid cellular proliferation directly, indirect effects are also possible. For example, severe hypercalcemia in the setting of humans with NSHPT or mice homozygous for knockout of the CaR might indirectly stimulate parathyroid cellular proliferation by reducing circulating levels of $1,25(OH)_2D$ (455), since the latter inhibits the proliferation of parathyroid cells, at least in some experimental systems (254). The intracellular mechanism(s) through which the CaR inhibits parathyroid cellular proliferation remains to be identified.

Another parameter of parathyroid function that is probably controlled by the CaR is the level of expression of the PTH gene. Several studies have established that high Ca_o^{2+} reduces the level of the mRNA for preproPTH (44, 394, 395, 413, 467). Garrett et al. (156) showed in preliminary studies that NPS R-568 decreases the level of PTH mRNA in bovine parathyroid cells, thereby suggesting that the CaR mediates this action of high Ca_o^{2+} on this parameter (156). High Ca_o^{2+} exerts numerous additional effects on parathyroid cells, such as modulating K^+ channels (108, 231, 270), stimulating the activity of the hexose monophosphate shunt (303), and increasing cellular respiration (173; for review, see Ref. 52). Additional studies are needed to determine which of these various actions of Ca_o^{2+} are mediated by the CaR and to identify the signal transduction pathways that are involved.

B. C Cells

Studies on the regulation of calcitonin secretion by high Ca_o^{2+} initially stressed that this process differed in a fundamental way from that through which Ca_o^{2+} regulates PTH release (132, 133, 147, 402). CT secretion, in contrast to PTH release, is stimulated by raising Ca_o^{2+} above its normal levels in the blood in association with increases in Ca_i^{2+}. The relationship between increases in Ca_i^{2+} and activation of CT secretion is similar to the more classical, positive relationship between Ca^{2+} and exocytosis that is observed in most other secretory cells (52, 127). Furthermore, influx of Ca_o^{2+} via voltage-dependent calcium channels is the predominant contributor to high Ca_o^{2+}-evoked increases in Ca_i^{2+} in C cells (133, 147, 305, 402). In parathyroid cells, in contrast, mobilization of Ca_i^{2+} is a major factor contributing to high Ca_o^{2+}-evoked increases in Ca_i^{2+}, particularly immediately after raising Ca_o^{2+} (315, 319). The patterns of the high Ca_o^{2+}-elicited increases in Ca_i^{2+} are also different in these two cell types. Single C cells often show oscillations in Ca_i^{2+} (132, 133), which are either much less frequent (296) or are not observed in single parathyroid cells (133). Finally, most of the high Ca_o^{2+}-induced influx of Ca_o^{2+} into C cells occurs through voltage-sensitive Ca^{2+} channels, as noted above, while considerable uncertainty remains concerning the channels through which uptake of Ca_o^{2+} takes place in parathyroid cells. The latter are most likely NCCs permeable to Ca^{2+} (52, 80, 305, 352). Thus it was generally assumed that the mechanisms mediating Ca_o^{2+} sensing in parathyroid and C cells are distinctly different, with the latter likely involving some form of voltage-sensitive Ca^{2+} channel.

Studies utilizing Northern analysis, in situ hybridization, RT-PCR with sequencing, and/or immunohistochemistry with anti-CaR antisera have convincingly demonstrated, however, that C cells contain the same CaR that is present in parathyroid and kidney cells (146, 158). Nevertheless, not all CaR agonists that modulate PTH secretion and other parameters of parathyroid function elicit CT secretion from C cells. For instance, while raising Mg_o^{2+} inhibits PTH secretion, Mg_o^{2+} has little or no effect on CT secretion from sheep parafollicular cells (288). Recent studies using sheep C cells have suggested the following model for how the CaR activates voltage-sensitive Ca^{2+} channels and CT secretion: high Ca_o^{2+}-evoked, CaR-mediated stimulation of PC-PLC provides a source of diacylglycerol for the PKC-induced activation of a NCC. The latter permits entry of Na^+ and Ca^{2+} into the cells, which produces cellular depolarization and subsequent activation of voltage-gated, principally L-type Ca^{2+} channels (288). The resultant rise in Ca_i^{2+} stimulates secretion of CT. The CaR likely also regulates other aspects of C-cell function, including activating a pertussis toxin-sensitive, PKC-mediated acidification of serotonin-con-

taining secretory vesicles (the stimulation of secretion of serotonin and CT by high Ca_o^{2+}, in contrast, is insensitive to pertussis toxin) (429). This acidification of secretory vesicles is thought to play a key role in their loading with serotonin and neurotransmitters or hormones.

C. Kidney

Microdissection of short (1–2 mm) segments of rat kidney tubules, followed by isolation of RNA and subsequent use of RT-PCR with rat CaR-specific primers has clarified the distribution of CaR transcripts along the nephron of this species. Riccardi et al. (388) utilized this approach to demonstrate that CaR mRNA is present along essentially the entire nephron, e.g., glomerulus, proximal convoluted (PCT) and proximal straight tubule (PST), MTAL, CTAL, distal convoluted tubule (DCT), cortical collecting duct (CCD), and IMCD. Several inaccessible segments that could not be studied for the presence of CaR transcripts in this manner were the thin descending and ascending limbs of the loop of Henle and the connecting segment between the DCT and CCD (380). A subsequent study carried out using a similar, albeit somewhat less sensitive, methodology confirmed the expression of CaR transcripts in MTAL, CTAL, DCT, and CCD but not in the other nephron segments that Riccardi et al. (380) found to express CaR mRNA (469). Rather than using Southern blot of the PCR products with a CaR-specific probe to identify transcripts for the receptor (380), Yang et al. utilized visual detection of PCR products combined with restriction digestion to confirm their identify as being CaR-derived (469). Given the results of immunohistochemistry with CaR-specific antisera to assess the receptor's expression along the nephron (see below), it appears that the differences between the results of these two studies are most likely accounted for by a lower sensitivity of the methodology in the latter for detection of the CaR's transcripts.

The studies performed to this point using immunohistochemistry with CaR-specific antisera have documented the localization of CaR protein in the proximal tubule (379), MTAL (379), CTAL (71, 379), DCT (379), and CCD (379) as well as IMCD (71, 401). In the proximal tubule, the CaR is located predominantly, if not exclusively, at the base of the brush border of the apical membrane of the tubular epithelial cells (379). The CaR also exhibits principally an apical distribution in IMCD (71, 401). In contrast, the CaR protein in CTAL is present at high levels on the basolateral surface of the epithelial cells (71, 379). It is likewise present predominantly on the basolateral side of the epithelial cells in the MTAL and DCT, albeit at lower levels (379). Within the CCD, the CaR is expressed in some, but not all, of the type A intercalated cells, which participate in acid-base homeostasis (379).

Knowing the precise location of the CaR along the nephron as well as the known actions of elevated levels of Ca_o^{2+} on tubular function have elucidated the receptor's functional significance in the kidney, although in many cases, proof of the receptor's involvement is lacking. Limited data are available on the regulation of the activity of various renal transporters by the CaR. In the proximal tubule, high levels of Ca_o^{2+} inhibit the Na^+-K^+-ATPase (263), an effect that likely contributes to the "diuretic" action of severe hypercalcemia in vivo. The receptor is located in the apical plasma membrane of the epithelial cells of the proximal tubule (379), where it lies in close proximity to the numerous transporters of various nutrients and electrolytes that reside within this portion of the nephron. The sodium-phosphate cotransporter, NPT2, which reabsorbs the bulk of the filtered phosphate in the proximal tubule (307), is one such transporter that could potentially be modulated by the CaR. The localization of the CaR within the proximal tubule of the rat kidney (379) is similar to that of NPT2 (308), both being located in the apical brush-border membrane as well as in subapical vesicles that likely participate in modulating cell surface expression of the cotransporter via changes in exocytosis and/or endocytosis (307–309). PTH, for example, promotes phosphaturia by stimulating the retrieval of NPT2-containing vesicles from the proximal tubular apical membrane by endocytosis (242) followed by lysosomal degradation of the cotransporter (348). It remains to be determined whether the CaR and NPT2 are actually colocalized and whether the former regulates the activity and/or trafficking of the latter. One possible mechanism for the reduction in serum phosphate in hypoparathyroid individuals whose serum calcium concentration is raised toward normal by treatment with vitamin D and calcium supplementation (419) could be a CaR-induced inhibition and/or removal of NPT2 from the apical membranes of the proximal tubule. Indeed, activation of PKC, as might be anticipated with high Ca_o^{2+}-evoked stimulation of the CaR, is known to promote retrieval of NPT2 (143).

Additional clues regarding the CaR's roles in regulating more distal segments of the nephron are provided by studies utilizing "experiments-in-nature" afforded by disorders of mineral ion homeostasis caused by inactivating or activating CaR mutations (49, 181). Earlier work had shown that raising peritubular levels of Ca_o^{2+} or Mg_o^{2+} decreases the tubular reabsorption of both ions in perfused segments of the TAL (358–360). The reabsorption of Ca^{2+} and Mg^{2+} in the CTAL are thought to occur principally via the paracellular pathway; their reabsorption is driven by the lumen-positive, transepithelial potential gradient that is generated by the transport of Na^+, K^+, and Cl^- by the apical Na^+-K^+-$2Cl^-$ cotransporter of the tubular epithelial cells combined with the recycling of K^+ into the tubular lumen through an apical K^+ channel (Fig. 4) (for reviews, see Refs. 123, 181). PTH and other hormones

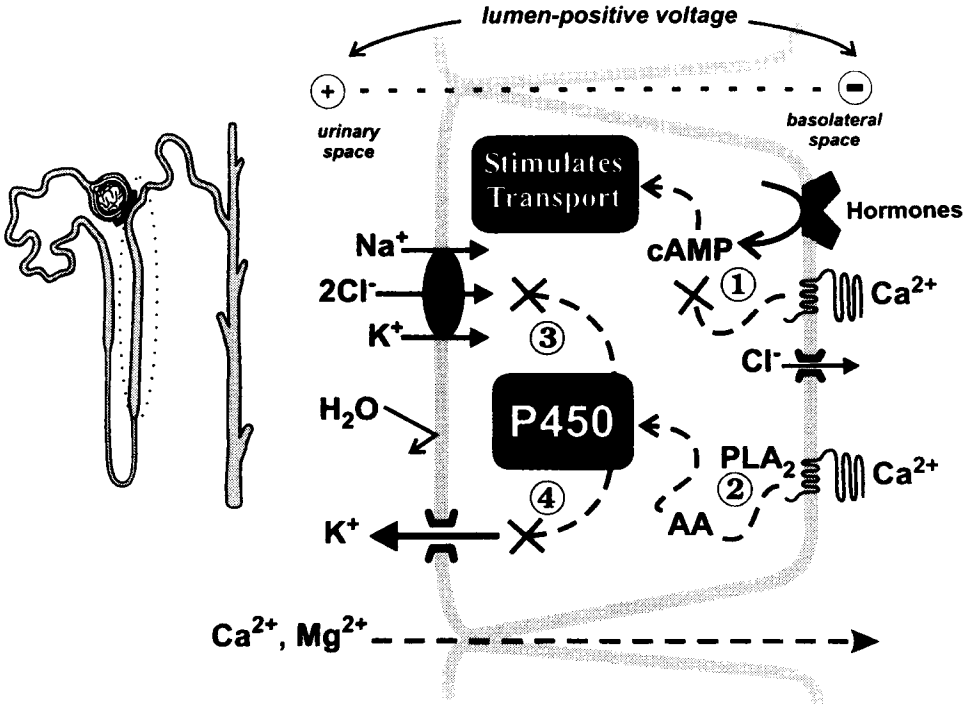

FIG. 4. Diagram showing schematically how the CaR likely regulates intracellular second messengers and ion transport in the renal cortical thick ascending limb. Hormones stimulating cAMP accumulation, such as parathyroid hormone, activate the paracellular reabsorption of Ca^{2+} and Mg^{2+} by stimulating the activity of the Na^+-K^+-$2Cl^-$ cotransporter as well as an apical K^+ channel and, in turn, the lumen-positive, transepithelial potential. The CaR, located similarly on the basolateral membrane, stimulates arachidonic acid (AA) production through direct or indirect activation of phospholipase A_2 (PLA_2) (*2*), which is metabolized via the cytochrome *P*-450 pathway to an active metabolite inhibiting the apical K^+ channel (*4*) and, perhaps, the cotransporter (*3*). Both actions reduce overall cotransporter activity, thereby reducing the lumen-positive potential and paracellular transport of divalent cations. The CaR probably also directly or indirectly (by raising Ca_i^{2+}) inhibits adenylate cyclase (*1*) and causes hormone-stimulated divalent cation transport as a result. (From Brown EM and Hebert SC. Calcium-receptor regulated parathyroid and renal function. *Bone* 20: 303–309, 1997.)

that enhance cAMP accumulation (e.g., glucagon, β-adrenergic catecholamines, and calcitonin) stimulate the reabsorption of Ca^{2+} and Mg^{2+} by increasing the cotransporter's overall activity and pari passu the magnitude of the lumen-positive potential (123, 181). Studies utilizing the patch-clamp technique have demonstrated that high Ca_o^{2+} and neomycin (another CaR agonist) reduce the activity of the apical K^+ channel by a mechanism involving a metabolite(s) of arachidonic acid generated by the cytochrome *P*-450 pathway, probably 20-hydroxyeicosatetraenoic acid (Fig. 4) (449, 450). If apical cycling of K^+ is reduced, luminal levels of K^+ decrease, the activity of the cotransporter likewise diminishes, and, therefore, paracellular transport of Ca^{2+} and Mg^{2+} decreases as well (181).

Recent studies have suggested that there are additional or alternative mechanisms through which the CaR modulates the function of the thick ascending limb and that there may be differences among species in these mechanisms. In perfused tubules from the CTAL of rat kidney, for instance, studies utilizing inhibitors or activators of PKA have suggested that the actions of high Ca_o^{2+} on net chloride flux are mediated by CaR-evoked changes in cAMP accumulation (118). In these studies, there was no effect of inhibitors of the cytochrome *P*-450 pathway on net chloride flux (118); the basis for the apparent discrepancy between the latter results and those of Wang et al. (450) requires further study. In contrast to the parathyroid cell, inhibition of cAMP accumulation in rat CTAL appears to involve stimulation of cAMP hydrolysis as well as pertussis toxin-insensitive inhibition of a Ca^{2+}-inhibitable isoform of adenylate cyclase owing to a high Ca_o^{2+}-induced, presumably CaR-mediated increase in Ca_i^{2+} (119). The latter results both from influx of Ca_o^{2+} (119) and mobilization of intracellular calcium stores (79, 119). In CTAL of rabbit kidney, in contrast, high Ca_o^{2+}-induced increases in Ca_i^{2+} occur through a mechanism that does not appear to involve activation of PI-PLC (124) but rather Ca_o^{2+} influx through basolateral Ca^{2+} channels that are sensitive to verapamil and nifedipene. The results in rabbit and rat CTAL both differ from those in mouse CTAL, in which high Ca_o^{2+} inhibits cAMP accumulation in association with inhibition of the reabsorption of Ca^{2+} and, to a lesser extent Mg^{2+}, but not NaCl (125).

It is likely that the CaR participates in the regulation of Ca^{2+} reabsorption not only in CTAL but also in DCT and the connecting segment (which lies between DCT and CCD), but very little is presently known about the CaR's role in these latter nephron segments. Bapty et al. (19), however, have recently demonstrated expression of the CaR in a murine cell line derived from DCT and have shown that elevating Ca_o^{2+} or Mg_o^{2+} raises Ca_i^{2+} and inhibits adenylate cyclase in this cell line. The second of these two actions clearly could inhibit PTH-stimulated reabsorption of Ca_o^{2+} in this nephron segment (148). Therefore, as in the CTAL (181), the CaR and PTH receptor in DCT and/or the connecting segment could have mutually antagonistic actions on the reabsorption of Ca^{2+}.

Regardless of the precise mechanism(s) through which it modulates renal tubular handling of divalent cations, the decreased capacity of persons with inactivating mutations of the CaR to increase their urinary excre-

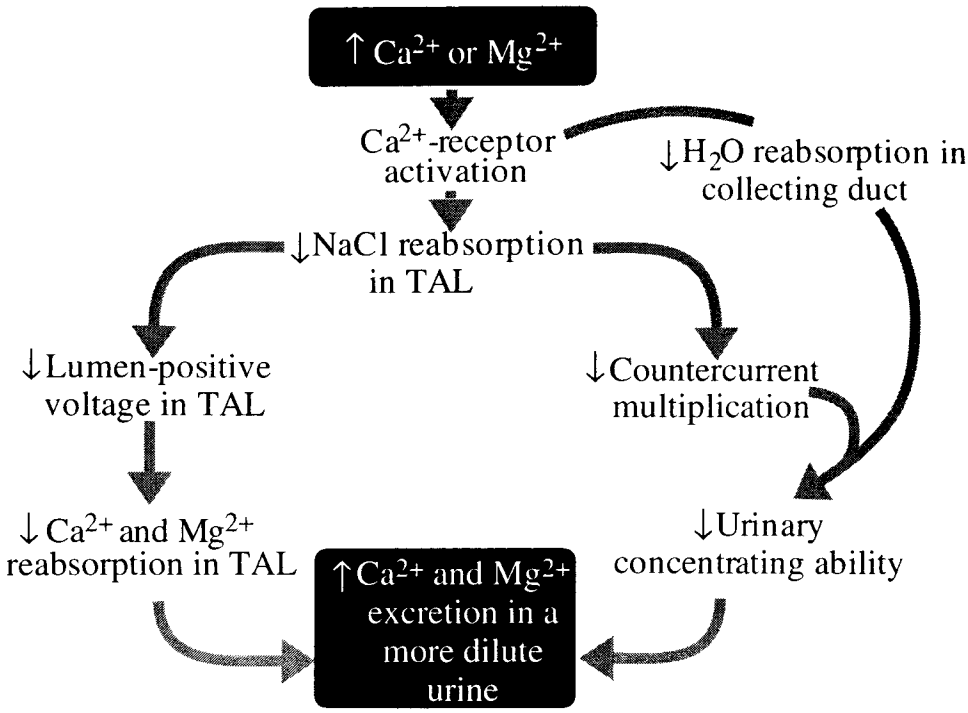

FIG. 5. Hypothetical mechanisms that could coordinate systemic calcium and water homeostasis in humans. See text for details. The *top panel* illustrates renal mechanisms through which the CaR may inhibit maximal urinary concentrating capacity. TAL, thick ascending limb. (From Brown EM and Hebert SC. Novel insights into the physiology and pathophysiology of Ca^{2+} homeostasis from the cloning of an extracellular Ca^{2+}-sensing receptor. *Regulatory Pept Lett* 7: 43–47, 1997.)

tion of Ca^{2+} in response to hypercalcemia provides indirect support for the CaR's role in this process (11, 49, 117, 181). In contrast, individuals with activating mutations of the CaR exhibit excessively high levels of urinary Ca^{2+} excretion at any given level of serum Ca^{2+} in the untreated state, presumably owing to the presence of activated CaRs along in the kidney, especially in CTAL (20, 344).

Hypercalcemic patients, particularly those with severe hypercalcemia (greater than ~14 mg/dl), not infrequently have abnormally decreased urinary concentrating capacity and, occasionally, frank nephrogenic diabetes insipidus (160, 422). The CaR's presence in segments of the nephron that participate in urinary concentration (379, 401) has afforded novel insights into the likely mechanism(s) underlying the long-recognized but poorly understood effects of high Ca_o^{2+} on this parameter of renal function. As noted previously, perfusing isolated rat IMCD tubules with high Ca_o^{2+} or neomycin, probably by activating CaRs present in their apical membrane, reversibly inhibits vasopressin-elicited, transepithelial water flow by ~35–40% (401). The CaR is also present within the same apical endosomes that contain the vasopressin-regulated water channel, aquaporin-2. This observation raises the possibility that the CaR reduces vasopressin-stimulated water flow in this nephron segment by stimulating the endocytosis and/or inhibiting the exocytosis of these endosomes out of or into the apical plasma membrane, respectively (401). Furthermore, chronic hypercalcemia in rats induced by treatment with vitamin D causes reduced expression of the aquaporin-2 water channel (400), which would further reduce vasopressin-stimulated water flow in the terminal collecting duct. High Ca_o^{2+}-induced, CaR-mediated inhibition of NaCl reabsorption in the MTAL (449, 450) would also diminish the magnitude of the medullary countercurrent gradient, which would be expected to reduce further the maximal urinary concentrating power of hypercalcemic persons (Fig. 5). Interestingly, individuals with inactivating mutations of the CaR are able to concentrate their urine normally despite their hypercalcemia (282), probably because they are resistant to the usual inhibitory effects of Ca_o^{2+} on the urinary concentrating mechanism. Conversely, persons with activating mutations of the CaR can develop symptoms of diminished urinary concentrating capacity at normal or even low levels of Ca_o^{2+} when treated with vitamin D and calcium supplementation, presumably because their renal CaRs are overly sensitive to the usual actions of raising Ca_o^{2+} on the urinary concentrating system (344). The CaR likely has additional functional roles in the collecting duct given its expression in some type A intercalated cells (379), but additional studies are needed to determine whether, for example, it directly modulates renal acid-base handling, one of the key functions of this cell type.

What are the physiological implications of the defective renal handling of water in hypercalcemic patients? We previously suggested that this action of high Ca_o^{2+} affords a mechanism that integrates the renal handling of divalent cations, particularly Ca^{2+} and water, thereby permitting appropriate "trade offs" in how these parameters of renal function are regulated under specific physiological conditions (181). For example, when a systemic

calcium load must be disposed of, there is a CaR-mediated increase in urinary Ca^{2+} content owing to reduced reabsorption tubular of Ca^{2+} in the CTAL, and perhaps the DCT. The resultant rise in luminal levels of Ca_o^{2+} in IMCD, particularly in a dehydrated individual, might predispose to the formation of Ca^{2+}-containing renal stones were it not for the concomitant inhibition of maximal urinary concentrating capacity in MTAL and IMCD.

Furthermore, abundant CaRs are present in the subfornical organ (SFO) (388), which is an important hypothalamic thirst center (415). These CaRs may provide an additional layer of integration of Ca_o^{2+} and water homeostasis, as follows: a high Ca_o^{2+}-induced, CaR-mediated increase in thirst with a resultant increase in drinking could prevent dehydration that might otherwise be the consequence of a fixed renal loss of free water because of concomitant resistance of the kidney to vasopressin (Fig. 5). Finally, prior studies have documented the existence of a specific "calcium appetite" in rats (433) that could provide a mechanism for a physiologically appropriate modulation of the intake of calcium-containing food during hypo- and hypercalcemia. We postulate, therefore, that multiple layers of CaR-mediated integration and coordination participate in the regulation of water and Ca^{2+} metabolism, serving to optimize the capacity of terrestrial organisms to adapt to their intermittent access to dietary Ca_o^{2+} and water (181). Thus in addition to integrating multiple physiological signals, as described in section IX, the CaR likely participates in the coordination of several of the body's homeostatic systems (e.g., for Na^+, Ca^{2+}, Mg^{2+}, and water).

D. Osteoclasts, Osteoblasts, and Osteocytes

1. Osteoclasts and their precursors

Recent studies have provided increasing evidence that the CaR is expressed in and could have important functional roles in bone cells and/or their precursors. For example, among cells of the osteoclast lineage, monocyte/macrophage-like cells express the CaR (464, 466). These CaR-expressing cells may serve as osteoclast precursors, since cells of this lineage are known to form mature multinucleated osteoclasts through a process of differentiation and fusion (278, 419). Indeed, a recent study identified the CaR in putative preosteoclasts in spleen-derived cultures that form multinucleated, osteoclast-like cells in vitro (230). These workers showed that high levels of Ca_o^{2+} as well as the calcimimetic CaR activator R-467 (albeit at very high concentrations) (320) inhibited the formation of osteoclast-like cells in vitro, suggesting a role for the CaR in regulating osteoclastogenesis. Another study suggesting a functional role for the CaR in mature osteoclasts demonstrated that polycationic CaR agonists, including extracellular Gd^{3+} and neomycin, activated an a NCC in rat osteoclasts and mobilized intracellular calcium stores (474). Several other studies, as noted previously, have suggested that the pharmacology of the osteoclast Ca_o^{2+}-sensing mechanism differs significantly from that of the CaR. Further studies are needed to resolve this point. A recent study, however, has shown that mature rabbit osteoclasts, in fact, express the CaR as assessed by Northern blot analysis and RT-PCR (226). Furthermore, raising the level of Ca_o^{2+} in the medium or addition of the polycationic CaR agonists extracellular Gd^{3+} or neomycin inhibited bone resorption by these rabbit osteoclasts in vitro (226), providing additional indirect evidence for a functional role of the CaR. It is important in a study such as this, however, with a potentially heterogeneous cell population, to utilize approaches that identify CaR transcripts or protein in individual cells. Thus the use of in situ hybridization and/or immunohistochemistry in future studies would rule out the possibility that CaR transcripts were actually present in some contaminating cell type. Therefore, while increasing evidence supports the CaR's expression and functional relevance in both osteoclast precursors and mature osteoclasts, additional confirmatory studies are needed. Moreover, further work is needed to determine whether the CaR can explain the known effects of high Ca_o^{2+} and other polyvalent cations on osteoclast function or whether there is, in fact, another ryanodine receptor-like Ca_o^{2+}-sensing mechanism expressed in these cells as well (469). Finally, in addition to studying the putative role of the CaR in regulating processes such as bone resorption and osteoclastogenesis in osteoclasts and/or their precursors, it will be important to determine whether it has additional important roles in these cells, such as modulating apoptosis and/or cytokine secretion (475).

2. Osteoblasts and their precursors

House et al. (204) showed that alkaline phosphatase-expressing cells derived from bone marrow, potentially representing osteoblasts or preosteoblasts, expressed the CaR, raising the possibility that this receptor is expressed in and plays functional roles in cells of the osteoblast lineage. In fact, elevated levels of Ca_o^{2+} have several physiologically relevant actions on osteoblast-like cells, including stimulating their proliferation and chemotaxis as well as modulating several intracellular second messenger systems (for more detailed discussions, see sect. IV*B* and Ref. 363). What is the evidence that osteoblasts and/or their precursors express the CaR? One group has consistently failed to find evidence for expression of the CaR in the osteoblast lineage and, instead, has suggested that osteoblasts express a distinct cation-sensing receptor that responds to extracellular Al^{3+} as one of its agonists [extracellular Al^{3+} is only a weak agonist of the CaR (417)] (362, 364, 365).

Two other groups, however, have provided evidence that the CaR is expressed in several osteoblast-like cell lines and is a candidate for mediating some or even all of the known actions of high Ca_o^{2+} on osteoblast function. Yamaguchi and co-workers have shown that CaR protein and mRNA are expressed in several osteoblast-like cells lines, including murine MC3T3-E1 cells (463) as well as the human SAOS-2 and the rat UMR-106 cell lines (465). These workers found that high levels of Ca_o^{2+} and other polycationic CaR agonists stimulate the proliferation and chemotaxis of MC3T3-E1 cells, indicating that the CaR could potentially mediate these actions of high Ca_o^{2+} on this cell line (463). Kanatani et al. (230) have recently confirmed the expression of CaR mRNA and protein in MC3T3-E1 cells (230). During their proliferative phase in culture, MC3T3-E1 cells resemble preosteoblasts; they subsequently cease dividing, express alkaline phosphatase and other markers of mature osteoblasts, and actually form mineralized bone nodules in vitro under appropriate conditions (463). Yamaguchi et al. (463) found that the CaR is expressed throughout the culture period as assessed by Western analysis, suggesting that the CaR may function in both preosteoblasts and mature osteoblasts. Finally, Chang et al. (83) have recently shown that both CaR mRNA and protein are present in slices of bovine, murine, and rat bone using in situ hybridization and immunohistochemistry, respectively.

The murine ST-2 stromal cell line also expresses CaR mRNA and protein, and high Ca_o^{2+} stimulates their proliferation and chemotaxis (462). Stromal cells can serve as precursors of osteoblasts and also produce mediators that impact on bone turnover by modulating the functions of osteoblasts and osteoclasts (278). Therefore, the CaR in stromal cells could participate in bone turnover either directly, if these cell differentiate to osteoblasts, or indirectly, by influencing this process via one or more mediators. In future studies it will be very important for additional laboratories to confirm these findings and to extend the use of similar approaches to identifying the CaR in bona fide osteoblasts (and, for that matter, osteoclasts) in intact bone. Furthermore, it will be essential to utilize genetic approaches [e.g., isolating bone cells from mice with knockout of the CaR (196) or utilizing dominant negative CaR constructs (15, 291)] or pharmacological methods [i.e., specific CaR agonists (320) and/or CaR antagonists (318)] to document that the CaR is not only expressed in bone cells but also mediates some or all of the known actions of Ca_o^{2+} on these cells.

3. Osteocytes

Not only osteoclasts and osteoblasts, but also osteocytes are responsive to changes in the level of Ca_o^{2+} (227, 228). The latter represent osteoblasts that have completed their role in bone formation and have become encased within the bone substance, where they extend processes into narrow canaliculi within the bone (212, 227, 370). They still, however, may participate in mineral ion homeostasis, perhaps sensing mechanical forces on bone (419). Kamioka et al. (228) showed that raising the level of Ca_o^{2+} increases Ca_i^{2+} in isolated chick osteocytes through a mechanism that involves mobilization of Ca^{2+} from its intracellular stores, presumably by some type of Ca_o^{2+}-sensing mechanism. The pharmacology of the effects of various divalent cations on Ca_i^{2+} in osteocytes, especially the fact that extracellular Ni^{2+} and extracellular Cd^{2+} evoke similar responses to those of Ca_o^{2+} (228), suggests that the Ca_o^{2+}-sensing mechanism in this cell type is more similar to that of the osteoclast than to the CaR (477, 479).

E. Chondrocytes

Although cartilage-forming cells (chondrocytes) are not directly involved in systemic mineral ion homeostasis, they play key roles in the formation and growth of the skeleton by providing a cartilaginous model of the future skeleton that is gradually transformed into bone. The growth plate represents a site where the process of bony replacement of the cartilaginous model of the future long bone enables longitudinal growth until the skeleton is fully mature at the end of puberty. The availability of Ca^{2+} is known to be important for ensuring the proper growth and differentiation of cartilage cells and attendant skeletal growth (208, 209, 378). Furthermore, alterations in Ca_o^{2+} modulate the differentiation and/or other functions of chondrocytes (33, 460), which arise from the same mesenchymal stem cell giving to osteoblasts, adipocytes, smooth muscle cells, and fibroblasts (37, 122). For these reasons, Chang et al. (82) utilized a rat cartilage cell line, RCJ3.1C5.18, to determine whether cells of this lineage express the CaR and whether the latter mediates various actions of Ca_o^{2+} on the function of these cells.

Raising the level of Ca_o^{2+} exerts several actions on this cell line, including producing dose-dependent reductions in the mRNAs encoding a major proteoglycan in cartilage, aggrecan, as well as the α_1-chains of types II and X collagen and alkaline phosphatase (82). RCJ3.1C5.18 cells expressed CaR transcripts as assessed by in situ hybridization and CaR protein as determined by immunocytochemistry and Western blot analysis. Furthermore, treatment of these cells for 48–72 h with an antisense oligonucleotide construct specific for the CaR lowered the level of the CaR protein substantially and promoted an associated increase in expression of aggrecan mRNA (82), consistent with a mediatory role of the CaR in regulating this gene. These results indicated, therefore, that *1*) Ca_o^{2+} modulates the expression of several biologically important genes expressed by this chondrocytic cell line, *2*) cells of the cartilage-forming lineage express the CaR, and

3) the receptor mediates some or all of these actions of Ca_o^{2+} in this cartilage cell model. Further studies are needed to assess the CaR's expression and biological role(s) in cartilage cells in vivo.

Therefore, the CaR is expressed not only in the cells that form and resorb bone as part of skeletal remodeling and systemic Ca_o^{2+} homeostasis but also in those that are involved in the processes of skeletal development and growth. Moreover, it regulates the functions of these cells in ways that appear to be biologically relevant. Additional studies are needed, however, using model systems such as the CaR knockout mice (196) or transgenic mice with knockout or overexpression of the CaR in osteoblasts, osteoclasts, osteocytes, or chondrocytes and/or their precursors to document further the importance of Ca_o^{2+} sensing by these various cells in vivo. Presumably, it is critical that not only the formation and resorption of bone in adult life but also the development and growth of the skeleton earlier in life be precisely coordinated so as to match the availability of mineral ions to their disposition in either the growing or mature but constantly remodeling skeleton. It will be of interest in future studies to determine whether similar mechanisms also exist for sensing and regulating the disposition of skeletal phosphate, the other major component of the mineral phase of bone.

F. Intestine

The major site of Ca^{2+} absorption is the duodenum, although substantial absorption of calcium also takes place in the proximal colon. The roles of $1,25(OH)_2D$, the intracellular calcium binding protein calbindin, and the basolateral calcium pump and sodium/calcium exchanger in intestinal Ca^{2+} absorption have been extensively investigated, although many details of this process remain to be fully understood (43, 138, 233, 336–338, 453). Calcium ions taken up across the apical (e.g., luminal) plasma membrane of the absorptive cell diffuse down their intracellular concentration gradient from the apical to the basolateral side of the cell using calbindin as a "shuttle," where they are then pumped out of the cell via the pump and exchanger. Until recently, a precise molecular characterization of the mechanism underlying apical uptake of calcium ions by the absorptive cells of the small and large intestine was lacking. We (345) and others (197, 198) have recently cloned and characterized calcium channel-like transporters that likely represent the major mediator(s) of the apical uptake of Ca^{2+} in the intestine as well as in the DCT and connecting segment of the kidney (197, 198).

Several cell types within both the small and large intestines, including the villus cells of the proximal small intestine (duodenum and ileum) and the surface epithelial cells of the proximal colon, both of which are involved in absorption of calcium, also express CaR mRNA and protein (85, 151). Thus the CaR could potentially directly or indirectly participate in mineral ion homeostasis via its actions on intestinal function, such as modulation of the absorption and/or secretion of calcium, but only limited information is available in this regard, and no such actions have been described to date. A more detailed discussion of the CaR's possible roles in regulating functions of the gastrointestinal tract unrelated to mineral ion homeostasis is provided in section XIII.

G. Placenta

During pregnancy, the placenta plays a key role in mineral ion metabolism of the fetus owing to the fact that all Ca^{2+} must be transported from the maternal to the fetal circulation through the placenta. Most of the fetal skeleton forms in the third trimester, and there is deposition of ~30 g of skeletal calcium during this time (385). Ca_o^{2+}-sensing cells have been shown to be present in the placenta and could potentially play some role(s) in regulating transport of Ca^{2+} between mother and fetus, perhaps by regulating the secretion of PTHrP by placental cells (188). As with parathyroid chief cells, high levels of Ca_o^{2+} raise the level of Ca_i^{2+} in human placental cytotrophoblasts (39, 223). Moreover, there is an inverse relationship between Ca_o^{2+} and PTHrP secretion in these cells, suggesting similarities in the mechanism(s) underlying Ca_o^{2+} sensing by parathyroid cells and cytotrophoblasts (188). The fetal parathyroid gland also secretes both PTH and PTHrP in utero, however, and it has been suggested that it may also contribute to regulating Ca^{2+} transfer across the placenta (385).

Recently, Bradbury et al. (40) have demonstrated that transcripts for the CaR are expressed in cytotrophoblast cells from human term placenta. In addition to transcripts that encode the full-length receptor protein, an additional, alternatively spliced, transcript is expressed not only in cytotrophoblasts but also in human parathyroid (40). This alternatively spliced RNA lacks exon 3 and encodes a truncated and very likely inactive receptor protein, since removal of this exon introduces a frame shift, thereby producing a premature stop codon within the receptor's ECD. The full-length CaR is also present in cytotrophoblasts, however, which makes it a candidate for mediating some or all of the known actions of Ca_o^{2+} on this cell type.

A careful study by Kovacs et al. (253), utilizing mice with knockout of the CaR and/or PTHrP and its receptor, has recently provided additional insights into the role of the CaR in maternal-fetal calcium metabolism. This work demonstrated that disruption of the CaR leads to an increase in the fetal calcium concentration of both CaR (+/−) (e.g., those heterozygous for CaR knockout) and (−/−) (i.e., homozygous) fetuses, although the level of

Ca_o^{2+} in the latter is not significantly higher than that in the former. Additional biochemical abnormalities included modest and marked increases in serum PTH and serum $1,25(OH)_2D$ levels in the (+/−) and (−/−) fetuses (indicative of hyperparathyroidism), respectively, reduced skeletal Ca^{2+} in the (−/−) fetuses accompanied by increased markers of bone resorption in amniotic fluid (documenting increased bone resorption) and elevated amniotic fluid Ca^{2+} in both (suggesting increased urinary Ca^{2+} excretion) (253). The increased levels of $1,25(OH)_2D$ in the (+/−) and (−/−) fetuses were thought to be secondary to the hyperparathyroidism, which, in turn, was caused by the reduced levels of CaR expression. The hyperparathyroidism also presumably caused the increased bone resorption, although it was not possible to rule out a contribution of a lack of the CaR in bone cells per se. The increased urinary Ca^{2+} excretion despite the loss of one or two CaR alleles presumably reflected the fact that CaR expression in the kidney is normally low in utero and only rises peri- and postnatally to its adult levels (84). Thus the loss of the CaR had no impact on renal calcium handling in utero, and the increased filtered load of Ca^{2+} caused by the attendant hypercalcemia was apparently largely excreted into the amniotic fluid by the "immature" fetal kidneys. Thus, in terms of fetal Ca^{2+} homeostasis, the presence of the CaR is essential for normal Ca_o^{2+}-regulated PTH release and, indirectly, for normal bone turnover and renal Ca^{2+} excretion (by ensuring normal fetal levels of PTH and serum Ca^{2+}) (253).

What was the impact of loss of the CaR in the fetus on placental Ca^{2+} transport in this model? Placental transport of Ca^{2+} was modestly reduced in (+/−) fetuses and substantially decreased in those that were (−/−). The greater reduction in this parameter in the (−/−) fetuses presumably contributed to the similarity in their levels of blood Ca_o^{2+} to those in the (+/−) fetuses despite their markedly higher levels of PTH. This difference in placental calcium transport was abolished by concomitant knockout of the PTHrP gene, suggesting that reduced placental transfer of Ca_o^{2+} owing to loss of the CaR might be mediated in part by reduced PTHrP-mediated calcium transfer. Thus the fetal CaR apparently contributes to the regulation of placental Ca_o^{2+} transfer in a fashion that is dependent on PTHrP, although the mechanism(s) by which it does so requires further investigation (253).

Juhlin and co-workers (195, 223, 272, 273) have provided evidence that gp330, the large Ca_o^{2+}-binding protein that binds Ca_o^{2+} but likely serves principally as an endocytic receptor, is expressed in cytotrophoblastic cells of the placenta. It remains to be determined whether this protein participates in maternal-fetal mineral ion homeostasis directly or perhaps indirectly by interacting in some manner with the CaR, since the two proteins are coexpressed in several tissues [e.g., parathyroid, placenta, and proximal tubule of the kidney (220)], and monoclonal antibodies directed against gp330 interfere with Ca_o^{2+} sensing by the parathyroid cell (221).

XI. THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR AND THE INTEGRATED CONTROL OF SYSTEMIC EXTRACELLULAR CALCIUM HOMEOSTASIS

In free-living terrestrial organisms, there is only intermittent dietary intake of Ca^{2+} from the environment (419). Therefore, tetrapods have evolved a complex homeostatic system that ensures a nearly constant level of Ca_o^{2+} in bodily fluids, which varies by only a few percent over the course of a day or even a lifetime (Fig. 6) (52, 419). This system affords great flexibility in its capacity to adjust the fluxes of Ca^{2+} between the extracellular fluid (ECF) and the environment via the kidneys and intestines as well as between the ECF and bone.

The egg-laying cycle of birds provides a remarkable example of how this system adjusts to large changes in the organism's needs for Ca^{2+}. A laying hen deposits an amount of Ca^{2+} in each egg that is on the order of 10% of what is present in the hen's entire skeleton. This Ca^{2+} must be acquired from dietary sources and from the skeleton over a few hours. Subsequently, however, the Ca^{2+} lost from the skeleton must be rapidly repleted, as often as on a daily basis, to avoid progressive depletion of skeletal Ca^{2+} over many egg-laying cycles. These large Ca^{2+} fluxes necessitate that all of the ionic Ca^{2+} in the plasma of the hen be turned over with a time constant on the order of minutes (206). Nonetheless, the hen's mineral ion homeostatic mechanism is capable of maintaining a nearly invariant serum ionized Ca^{2+} concentration (126, 205). Such precision in the control of Ca_o^{2+} is, of course, crucial to ensure constant availability of Ca_o^{2+} for vital processes such as hormonal secretion, cardiac contractility, and so forth.

To maintain near constancy of Ca_o^{2+} in tetrapods requires that specialized cells sense even minute fluctuations in blood calcium concentration (for review, see Ref. 52). In normal human beings, the coefficient of variation of the serum ionized calcium concentration about its mean value is 2% or less (339, 340), a reflection of the exquisite capacity of these cells to sense small changes in Ca_o^{2+}. Classical examples of Ca_o^{2+}-sensing cells involved in systemic Ca_o^{2+} homeostasis are the PTH-secreting parathyroid chief cells and the CT-secreting C cells, which secrete less and more, respectively, of these calciotropic hormones when Ca_o^{2+} rises (52). Figure 7*A* shows the steep sigmoidal, inverse relationship between blood levels of the intact, secreted form of parathyroid hormone, PTH-(1—84), and Ca_o^{2+} in normal persons (42). This curve is described quantitatively by the following four parameters (Fig. 7*B*) (51): the maximal secretory rate at low Ca_o^{2+}

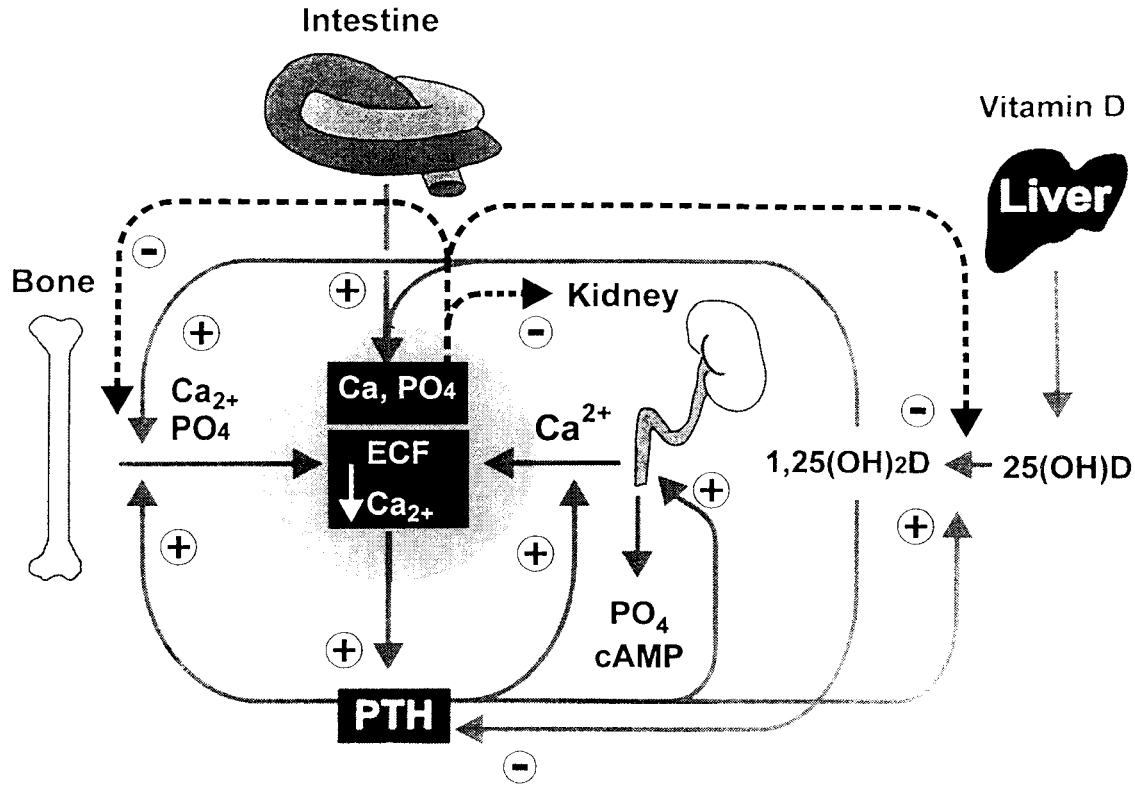

FIG. 6. Schematic diagram delineating the regulatory system that maintains Ca_o^{2+} homeostasis. The solid arrows and lines delineate the effects of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]; the dotted arrows and lines provide examples of how Ca_o^{2+} or phosphate ions produce direct actions on target tissues. Ca^{2+}, calcium; PO_4, phosphate; ECF, extracellular fluid; 25(OH)D, 25-hydroxyvitamin D; minus signs indicate inhibitory actions, whereas plus signs show positive effects. (From Brown EM, Pollak M, and Hebert SC. Cloning and characterization of extracellular Ca^{2+}-sensing receptors from parathyroid and kidney: molecular physiology and pathophysiology of Ca^{2+}-sensing. *Endocrinologist* 4: 419–426, 1994.)

(*parameter A*), the maximal slope at the curve's midpoint (*parameter B*), the midpoint or set point (e.g., the level of Ca_o^{2+} producing half-maximal suppression of PTH secretion) (*parameter C*), and the minimal secretory rate at very high levels of Ca_o^{2+} (*parameter D*). The set point is closely related to the normal value of Ca_o^{2+} in the blood, although the value at which the serum ionized calcium concentration is actually "set" is generally slightly higher than the parathyroid's set point. As a consequence, circulating PTH levels in vivo are ~20–25% of their maximal values at low Ca_o^{2+} (42). The steep slope of the curve between PTH and Ca_o^{2+} plays an important role in determining the range within which Ca_o^{2+} varies in vivo, because it ensures that even very small perturbations in Ca_o^{2+} produce large changes in PTH. The latter then normalizes Ca_o^{2+} by utilizing the mechanisms that are illustrated in Figure 6.

A steep sigmoidal relationship also exists between Ca_o^{2+} and CT release, but this curve is positive with respect to Ca_o^{2+} (12, 133, 402). CT can contribute to the maintenance of normality of Ca_o^{2+} because it has hypocalcemic actions, primarily by inhibiting osteoclastic bone resorption and stimulating renal Ca^{2+} excretion (419). Nevertheless, CT is not thought to contribute importantly to mineral ion homeostasis in adult humans, perhaps in part because of low prevailing rates of bone turnover that blunt the impact of its antiresorptive action. It does, however, exert powerful calciotropic actions in some species, particularly rodents, whose bones are constantly growing.

A third major calciotropic hormone is the active metabolite of vitamin D, 1,25$(OH)_2$D, whose production by the renal proximal tubular cells is stimulated by low levels of Ca_o^{2+} or phosphate in the blood as well as by increased circulating concentrations of PTH and reduced levels of 1,25$(OH)_2$D itself (280, 325, 419). The traditional view of the operation of the Ca_o^{2+} homeostatic system is as follows (Fig. 6): a decrement in the circulating level of Ca_o^{2+} as small as 1–2% elicits an increase in PTH secretion (Fig. 7). The latter acts rapidly (within minutes) on the kidney to enhance renal tubular Ca^{2+} reabsorption, thereby "resetting" the kidney to maintain a higher level of Ca_o^{2+}. PTH promotes a rapid phosphaturic response, which prevents the retention of phosphate mobilized from bone and absorbed by the intestine during later phases of the homeostatic response (52). PTH likewise acts on bone to increase the release of calcium and phosphate within 1–2 h

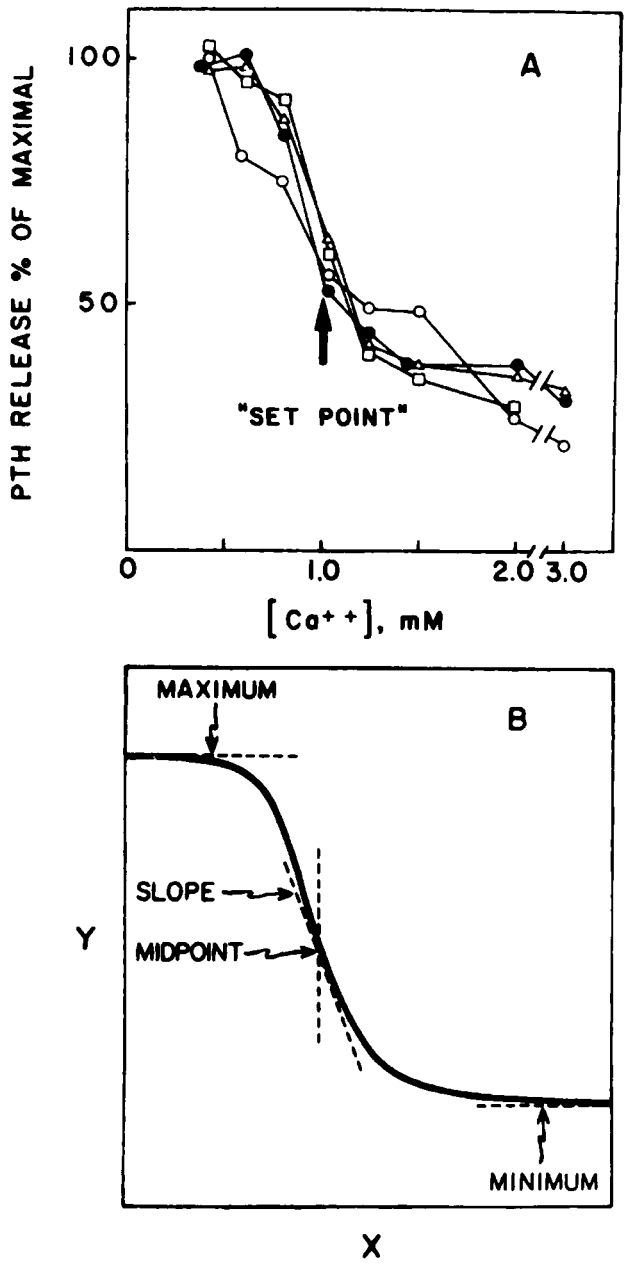

FIG. 7. *A*: the steep inverse sigmoidal function that relates PTH levels and Ca_o^{2+} in vivo. These curves were generated by infusing EDTA or Ca^{2+} in normal humans and measuring circulating levels of intact PTH as a function of serum ionized calcium concentration, here shown as millimolar levels. [Modified from Brown (52).] *B*: four-parameter model of the inverse sigmoidal relationship between extracellular calcium and PTH release based on $Y = \{(A - D)/[1 + (X/C)^B]\} + D$, where Y is the maximal secretory rate, B is the slope of the curve at its midpoint, C is the midpoint or set point, and D is the minimal secretory rate. [From Brown (51), Copyright The Endocrine Society.]

and, if hypocalcemia persists for several hours, enhances the renal synthesis of $1,25(OH)_2D$, thereby indirectly stimulating the absorption of calcium and phosphate from the intestine (52, 419). Increases in Ca_o^{2+} resulting from enhanced influx of Ca^{2+} from intestine and bone, combined with reduced urinary loss of Ca^{2+}, normalizes Ca_o^{2+} and returns the secretory rate of PTH to its basal level. Prolonged hypocalcemia of weeks to months duration can elicit additional adaptive responses of the homeostatic system, including parathyroid cellular hypertrophy and hyperplasia as well as increased recruitment of osteoclast precursors and generation of mature osteoclasts (419).

An important consequence of the cloning of the CaR has been the recognition that it is expressed not only in Ca_o^{2+}-sensing cells that secrete calciotropic hormones, e.g., parathyroid and C cells, but also in several other tissues involved in mineral ion metabolism, particularly the effector tissues acted upon by calciotropic hormones (61). In the kidney, as previously noted in section x*C*, the CaR mediates the known action of elevated levels of Ca_o^{2+} to promote increased urinary Ca^{2+} excretion, a homeostatically appropriate response (61, 181). The conversion by renal proximal tubular cells of 25-hydroxyvitamin D to $1,25(OH)_2D$ is likewise directly modulated by physiologically relevant changes in Ca_o^{2+} (455), with low Ca_o^{2+} stimulating and high Ca_o^{2+} inhibiting this conversion. Although the CaR is located in the proximal tubule [within the base of the brush border of the apical membrane of the tubular epithelial cells, as noted before (see sect. x*C*)], it is not yet known whether it mediates this action of Ca_o^{2+} on the production of $1,25(OH)_2D$. That this effect of Ca_o^{2+} on vitamin D metabolism is not indirect, mediated, for instance, by concomitant changes in circulating PTH levels, has been documented by studies in which circulating levels of parathyroid hormone were "clamped" by infusing PTH into parathyroidectomized rats via minipump (455). These animals still exhibited a steep inverse relationship between their levels of $1,25(OH)_2D$ in the blood and Ca_o^{2+} (455), not dissimilar from that between PTH and Ca_o^{2+} (Fig. 7). A similar inverse function relating Ca_o^{2+} to $1,25(OH)_2D$ has been noted in a boy with hypoparathyroidism, further emphasizing its independence from PTH (77). This relationship between vitamin D metabolism and Ca_o^{2+} is physiologically appropriate, because increasing levels of $1,25(OH)_2D$ during hypocalcemia stimulate the absorption of dietary Ca^{2+} and enhance bone resorption (419).

Additional cell types in which Ca_o^{2+}, in some cases known to be acting via the CaR, directly modulates functions of the mineral ion homeostatic system in physiologically relevant ways include osteoblasts and osteoclasts. As noted in section x*D*, the CaR could contribute to this action by reducing the formation of new multinucleated osteoclasts (230). Some data suggest that it can also inhibit the function of mature, bone-resorbing osteoclasts (226), although the pharmacology of the effects of various metal ions on the latter response in many studies has suggested the existence of some other type of ion-sensing receptor (469). As described in section x, elevated levels of Ca_o^{2+} also stimulate several aspects of osteoblast function in vitro (363) that could promote increased bone

formation in vivo and, therefore, reductions in Ca_o^{2+} (161, 201, 229, 363, 372, 421, 463).

This body of data strongly suggests that, in addition to participating in mineral ion homeostasis by regulating the secretion of the classical calciotropic hormones, PTH, CT, and $1,25(OH)_2D$, Ca_o^{2+} itself can act in effect as a local or systemic Ca_o^{2+}-regulating "hormone" (Fig. 6). In functions in this manner as the body's principal Ca_o^{2+}-lowering hormone, acting in part via stimulation of the secretion of CT but primarily through actions mediated by its own cell surface, Ca_o^{2+}-sensing receptor. This hormonelike role of Ca_o^{2+} modulates the function(s) of a variety of cells and tissues participating in mineral ion metabolism through changes in the local or systemic levels of Ca_o^{2+} that arise from the Ca^{2+}-translocating actions of these tissues. Although the CaR may be an important mediator of these actions of Ca_o^{2+}, there may well be additional Ca_o^{2+} sensors/receptors that contribute as well. The dashed lines in Figure 6 illustrate some of these direct actions of Ca_o^{2+} on tissues involved in mineral ion metabolism. Figure 6 also points out that extracellular levels of phosphate likewise exert direct actions on cellular function. It is likely that there is some type of phosphate-sensing mechanism in these cells (52), but the nature of the putative phosphate sensor remains obscure.

XII. EXTRACELLULAR CALCIUM-SENSING RECEPTOR-BASED THERAPEUTICS

The CaR represents an important target for the development of CaR-based therapeutics for the treatment of disorders in which the CaR is over- or underactive (316, 317). Although the development of such therapeutics has so far been directed at parathyroid disorders, it appears likely that it could extend to a wider variety of diseases of CaR-expressing tissues in which the CaR malfunctions or modulating the receptor's activity would have desirable therapeutic consequences (see sect. XIII for a discussion of the CaR's roles in "nonhomeostatic" tissues). Clinical studies are currently underway that are testing so-called "calcimimetic" CaR activators in the treatment of primary and secondary HPT (414). These agents are hydrophobic, low-molecular-weight drugs that allosterically activate the CaR (316, 320). They are ineffective in the absence of Ca_o^{2+}, but in the presence of Ca_o^{2+} or other polycationic CaR agonists, they enhance the receptor's apparent affinity for these agonists.

In primary HPT, calcimimetics produce rapid (within minutes) and substantial (>50%) reductions in circulating PTH levels that are followed several hours later by decreases in the serum calcium concentration, particularly at higher doses (414), because the drug resets the elevated set point of pathological parathyroid tissue toward normal. There is an initial increase in urinary Ca^{2+} excretion in patients with primary HPT during treatment with a calcimimetic that would have been anticipated, in part, as a result of the attendant rapid reduction in serum PTH. It is also possible that additional, direct actions of calcimimetics on CaRs in the CTAL and, perhaps, the DCT will lead to sustained hypercalciuria, but this effect has not been prominent to date. CaR agonists will also be very useful for treating uremic hyperparathyroidism, for which there are currently no fully effective forms of therapy. Conversely, a kidney-specific CaR antagonist might represent an effective mode of treatment for individuals with Ca^{2+}-containing renal stones. In the latter clinical setting, reducing the activity of CaRs in the CTAL would likely lower urinary Ca^{2+} excretion markedly (e.g., similar to that resulting from the renal Ca_o^{2+} resistance in FHH).

XIII. TISSUE DISTRIBUTION AND FUNCTIONS OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR IN TISSUES UNINVOLVED IN SYSTEMIC IONIC HOMEOSTASIS

A. Brain Cells, Including Neurons and Glia

In the adult rat brain, the CaR has been localized both by immunocytochemistry as well as by in situ hybridization with CaR specific antibodies and probes, respectively (86, 382, 386). The receptor is present at widely varying levels in numerous regions of the brain. The highest levels are found within the SFO and the olfactory bulbs. Substantial expression levels are also evident within the hippocampus, striatum, cingulate cortex, cerebellum, the ependymal zones of the cerebral ventricles, and perivascular nerves around cerebral arteries (87, 388, 392). Abundant levels of CaR expression within the SFO, which is an important hypothalamic thirst center (415), suggest that it may participate in the central control of systemic fluid and electrolyte balance, as noted in section X*C*. Thus, although mineral ion homeostasis is not often thought of as having central regulatory elements (i.e., in the brain), there are perhaps more complex relationships among the systems regulating mineral ion homeostasis and other homeostatic systems that are known to exhibit prominent neuroendocrine elements (i.e., water homeostasis) than recognized previously.

1. Neurons

The CaR is present within synaptic areas of all regions of the hippocampus. It is not presently known with certainty, however, whether its distribution is predominantly pre- and/or postsynaptic (87, 392). Its overall distribution is similar to those of the mGluRs and iGluRs (e.g., the NMDA receptor), which both have important

roles in certain types of long-term potentiation (LTP). Thus it is of interest that there are substantial increases in the expression of the CaR in the developing rat hippocampus at the time (during the first several weeks of life) when the development of the brain is progressing rapidly and LTP can first be induced (87). The function(s) that the receptor plays during this period of time, however, is essentially unknown (87, 89). The expression of the CaR in the granule and Purkinje cells of the cerebellum also suggests that it could have some type of role(s) in modulating cerebellar function, but no studies on the CaR's functions in these two cell types have been presented to date. The availability of brain tissue from mice that have targeted disruption of the CaR gene should eventually enable studies of whether the CaR participates in any such postulated roles; unfortunately, the limited viability of the homozygous CaR knockout mice complicates such investigations (196).

How could the CaR modulate the functions of cerebellar, hippocampal, and other types of neurons? Recently, we have shown that the CaR stimulates the activity of Ca^{2+}-permeable NCCs in several cell types, including hippocampal pyramidal neurons from rats and mice (471, 472). The relationship of this NCC to iGluRs, such as the NMDA channel, is not yet understood. CaR-mediated stimulation of this Ca^{2+}-permeable cation channel, however, could contribute to the increases in Ca_i^{2+} known to be necessary for induction of synaptic plasticity and would also be expected to increase neuronal excitability. It is possible that the depolarization induced by CaR-mediated activation of NCCs, owing to the influx of Ca^{2+} and Na^+, could secondarily activate voltage-sensitive Ca^{2+} channels, as is thought to occur in sheep parafollicular cells (284). Such an effect of CaR activation on neurons, however, has not been reported to date. In addition to activating neuronal NCCs, the CaR also enhances the activity of a Ca^{2+}-activated K^+ channel present in hippocampal pyramidal neurons from wild type but not in those from CaR knockout mice (441). In contrast to the effect of activating NCC, CaR-induced activation of calcium-activated potassium channel (CAKC) would be expected to reduce neuronal excitability. Thus the CaR modulates the activities of ion channels that contribute to the overall state of neuronal excitability. As discussed in more detail in section XIV, neuronal activation is often associated with reductions in Ca_o^{2+} as a result of activation of Ca^{2+}-permeable influx pathways. The ensuing reduction in Ca_o^{2+} could, therefore, reduce the degree of activation of NCCs mediated by the CaR and perhaps provide a negative-feedback mechanism for avoiding excessive elevations in neuronal Ca_i^{2+}. In contrast, in neurons in which the CaR couples strongly to CAKC, it could participate in a positive feedback mode; that is, decreased activity of the receptor during neuronal activity-dependent lowering of Ca_o^{2+} could reduce the CaR-mediated activation of CAKC and resultant cellular hyperpolarization, thereby enhancing neuronal excitability.

We showed recently that the CaR is present at robust levels in the enteric nervous system along essentially the entire gastrointestinal tract, being expressed both in Aurbach's plexus (e.g., between the circular and longitudinal smooth muscle layers in the intestinal wall) and Meissner's plexus (within the submucosa) (85, 98). Both plexi participate in the regulation of key intestinal functions, including motility, secretory, and absorptive activities. Interestingly, changes in Ca_o^{2+} affect many of these processes, especially motility. For instance, hypercalcemic patients not infrequently complain of constipation, while those with hypocalcemia may have symptoms of increased gastrointestinal motility (419). Additional effects of raising Ca_o^{2+} include increased gastrin release (374) and enhanced gastric acid secretion (23, 27, 142). All of these actions of Ca_o^{2+} could be CaR mediated (see below).

Finally, Bukoski et al. (66) have demonstrated that the CaR is expressed in perivascular sensory nerve endings in rat mesenteric artery. Subsequent studies of sensory nerve endings in other vascular beds revealed the following distribution of the CaR in terms of its apparent density in various beds: mesenteric branch artery > basilar artery = renal interlobar artery > main renal trunk artery > left anterior descending coronary artery (451). Additional work has documented that stimulation of the CaR in these nerve endings releases a vasodilatory substance, which is most likely an endogenous cannabinoid, such as *N*-arachidoylethanolamine (anandamide), that acts on a cannabinoid receptor within the vascular wall (207). As described in section XIV, there could potentially be sufficiently large changes in the level of Ca_o^{2+} in the interstitial fluid within a contracting muscle to modulate the activity of CaRs in the immediate vicinity (4).

2. Oligodendroglia

Not only neurons, but also oligodendrocytes express the CaR (92). In the latter cell type, the receptor stimulates cellular proliferation and the activity of a CAKC (92). Very little is known about how the CaR regulates the function of oligodendrocytes in vivo. It is of interest, however, that expression of the receptor increases severalfold during the first several weeks of postnatal life in the rat, a time when myelin formation is proceeding rapidly in the developing brain. It is conceivable, therefore, that the increase in CaR expression in oligodendroglia during this time could contribute indirectly (e.g., by increasing cellular proliferation) or directly (by currently unknown mechanisms) to formation of myelin during brain development.

The expression level of the CaR subsequently falls to a lower and stable level in adult rats. Subsequent to their role in myelin formation, oligodendroglia are thought to participate importantly in local ionic homeostasis within

the brain ECF. The CaR present in these cells could potentially contribute to such local homeostatic control. For example, because neuronal activity-dependent decreases in Ca_o^{2+} are accompanied by elevations in extracellular K^+, reduced activity of oligodendroglial CaRs could lower the activity of CAKC in these cells, thereby mitigating further increases in extracellular K^+.

3. Astrocytes

We have recently shown that the CaR is expressed in the astrocytoma cell line U87, where it stimulates the activity of a CAKC (93). Although an earlier study failed to detect expression of the CaR in primary astrocytes isolated from rat brain (392), we have recently demonstrated the presence of CaR mRNA and protein in primary human astrocytes (86). The function of the CaR in normal astrocytes is currently unknown.

4. Microglia

Microglia are the brain's macrophages. Because the CaR is expressed at robust levels in most circulating monocytes (466), it is not surprising that the CaR is also readily detectable in rat primary microglia (91), where, as in most other brain-derived cells studied to date, it activates a CAKC. Characterization of the function of the receptor in microglia requires further study. It is of interest, however, that Aβ peptides are known to modulate Ca_i^{2+} in microglia (252) and to be chemotactic for these cells (275), effects that could be CaR mediated (421, 466). Thus it is possible that CaR-mediated chemotaxis of microglia could participate in the inflammatory reaction that can be present in the vicinity of amyloid plaques in Alzheimer's disease.

B. Lens Epithelial Cells

High Ca_o^{2+} has a number of actions on cultured lens epithelial cells, including disrupting plasma membrane integrity, promoting loss of the cytoskeletal protein, vimentin, and stimulating Ca^{2+}-ATPase, effects that could contribute to cataract formation (For review, see Ref. 120). Furthermore, clinical findings, viz., the development of cataracts in individuals with hypoparathyroidism and the high Ca^{2+} content noted in their cataracts, suggest that Ca_o^{2+} (and potentially the CaR) could participate in the physiology and pathophysiology of lens epithelial cells (129). We recently demonstrated that cultured human lens epithelial cells express CaR mRNA and protein (90). The receptor stimulates the activity of a CAKC with a unitary conductance of ~82 pS. In view of the key role of Ca_o^{2+} in maintaining lens integrity, it is conceivable that the CaR contributes to local ionic homeostasis within the lens. Examining the properties of the lens and of lens epithelial cells from mice with knockout of the CaR gene (196) might provide additional useful information in this regard.

C. Pituitary Gland

RNA isolated from mouse and rat pituitary expresses transcripts for the CaR as assessed by RT-PCR (131), and murine pituitary-derived, ACTH-secreting AtT-20 cells express a functional CaR that mediates stimulation of ACTH secretion (131, 139). Elevating Ca_o^{2+} within a physiologically relevant range (e.g., 10–15% above the upper limit of normal) also stimulates ACTH release in vivo in normal volunteers (149, 444) as well as in persons being treated for psychiatric disorders with lithium (172). Furthermore, modulating the level of Ca_o^{2+} in vivo in humans has been found to alter the circulating levels of several other pituitary hormones, e.g., increasing gonadotropin hormone-releasing hormone-stimulated follicle-stimulating hormone and luteinizing hormone levels and inhibiting thyrotropin-releasing hormone-stimulated thyrotropin-stimulating hormone and prolactin secretion (444).

Functional evidence for CaR expression in the normal bovine pituitary gland has been provided by Shorte and Schofield (410), who showed that polycationic CaR agonists mobilized intracellular stores of calcium in the majority of dispersed pituitary cells. The expression of the CaR has recently been directly shown in growth hormone (GH)-secreting pituitary adenomas, in which the receptor increased Ca_i^{2+} and cAMP accumulation (390), as it does in AtT-20 cells (131). Although agonists of the CaR did not stimulate the release of growth hormone from these tumors in vitro in the absence of additional secretagogues, they did enhance growth hormone-releasing hormone-stimulated secretion of GH (390). Thus the CaR acts as a coagonist in this system. Although it is not known why the CaR is expressed in normal pituitary cells, let alone in these tumors, it would be of interest to determine whether the portal blood that supplies the releasing hormones to the pituitary gland from the hypothalamus undergoes changes in its level of Ca_o^{2+} as a function of alterations in the secretion of hypothalamic releasing factors. Secretory vesicles are known to contain high levels of Ca_o^{2+}, in some cases as high as 200 mM (322). Therefore, it is conceivable that Ca_o^{2+} could be secreted along with releasing factors from the hypothalamus and serve as a coagonist, as it does in vitro for the secretion of GH from GH-secreting adenomas (390). Moreover, further studies are needed to define which cell type(s) within the normal pituitary express the CaR.

D. Bone Marrow and Peripheral Blood

We recently showed that several cell types within the bone marrow express the CaR, including megakaryo-

cytes, erythroid progenitors, myeloid precursors, and cells with the morphological appearance of monocyte/macrophages (204). Of the cells of the various hematopoietic lineages that express the receptor in marrow, the only ones whose mature cells in peripheral blood contain the CaR are platelets and monocytes (204). We recently utilized CaR-specific antisera as well as RT-PCR to identify CaR protein and transcripts, respectively, in the majority (~85%) of peripheral blood monocytes (466), whereas Bornefalk et al. (35), who demonstrated that high Ca_o^{2+} stimulates secretion of IL-6 both in vivo and in vitro from peripheral blood monocytes, were unable to identify CaR transcripts in these cells using RT-PCR. The reason(s) for the failure to detect the CaR in the latter study is unclear.

Other studies that have examined the effects of physiologically relevant changes in Ca_o^{2+} on the functions of marrow-derived cells are scarce. Raising Ca_o^{2+} enhances the fusion of rat alveolar macrophages induced by $1,25(OH)_2D$ (215). Elevating Ca_o^{2+} also stimulates colony formation and increases Ca_i^{2+} in erythroid precursors obtained from uremic patients, an effect that was potentiated by $1,25(OH)_2D$ (76), perhaps because the latter upregulates the expression of the CaR (46). In the marrow, the CaR is expressed in hematopoietic precursors that probably experience significant alterations in the levels of Ca_o^{2+} to which they are exposed related to the prevailing state of bone turnover within the local bone/bone marrow microenvironment. In addition to the actions of Ca_o^{2+} on hematopoietic cells that were described above, it is also conceivable that the CaR could control other aspects of these cells' functions. For instance, because the CaR is expressed on erythroid and some myeloid precursors in the bone marrow but not on mature cells of those lineages in the peripheral blood (with the exception of monocytes) (204), perhaps it could control trafficking of these cells between marrow and peripheral blood. Finally, Ca_o^{2+} is known to modulate a variety of processes in platelets, including stimulating arachidonic acid release (287) and inhibiting cAMP accumulation (411); these effects could potentially be CaR mediated. Interestingly, Ca_o^{2+} has been quantified directly in platelet clumps formed as a result of platelet activation in vivo, and it decreases substantially (334). Thus changes in Ca_o^{2+} in the microenvironment to which platelets are exposed during clumping of these blood elements could perhaps both modulate their function(s) and also local levels of Ca_o^{2+} by regulating the fluxes of calcium ions between platelets and their immediate microenvironment.

High levels of Ca_o^{2+} promote the chemotaxis of several cell types, including monocytes (421), the murine monocytic cell line J774 (464), a bone marrow-derived, murine stromal cell line (ST-2) (462), and the murine osteoblastic cell line MC3T3-E1 (161, 463). Both J-774 cells and peripheral blood monocytes express robust levels of the CaR (466), making the receptor a good candidate for mediating this action of Ca_o^{2+}. CaR transcripts and protein are also expressed in ST-2 cells (462).

What is the physiological relevance of CaR-activated chemotaxis in these various cell types? In the case of osteoblasts and/or their precursors, the chemotactic response to high Ca_o^{2+} may provide a signal directing them to sites of ongoing bone resorption requiring replacement of the missing bone during the osteoblastic phase of bone turnover (363, 461). Furthermore, mononuclear cells with the appearance of monocytes/macrophages migrate to sites of bone resorption during the reversal phase of bone turnover interposed between the resorptive and formative phases (21). Sugimoto et al. (421) have shown that treatment of monocytes with elevated Ca_o^{2+} leads to the release of factor(s) that stimulate the expression of alkaline phosphatase (a marker of osteoblast differentiation) in MC3T3-E1 osteoblastic bone cells, while monocyte-conditioned medium inhibits the formation of multinucleated, putative osteoclasts in vitro. Therefore, high Ca_o^{2+}, by stimulating the release of cytokines or other factors [high Ca_o^{2+} is known to stimulate the release of IL-6 from peripheral blood monocytes (35)], could potentially serve to inhibit the formation of new osteoclasts and, at the same time, stimulate the differentiation of osteoblasts at sites of recent bone resorption.

High Ca_o^{2+} could also conceivably serve as a more generalized inflammatory signal for monocytes, macrophages, and their various tissue-specific forms (e.g., microglia and pulmonary alveolar macrophages). With the exception of red blood cells, which lack intracellular organelles, all other types of cells have levels of total intracellular calcium that are substantially higher than that in the blood owing to the presence of high concentrations of calcium within intracellular stores, e.g., the ER and secretory vesicles (384). In the case of smooth muscle, total cellular calcium is 8.3 mmol/kg wet weight, which is nearly half that of mineralized bone (384). Therefore, death of cells and release of cellular calcium in a soluble form could lead to substantial local increases in Ca_o^{2+}. In addition, high Ca_o^{2+} induces the fusion of pulmonary alveolar macrophages to form multinucleated giant cells (215), which could also potentially represent part of an integrated, CaR-mediated inflammatory reaction initiated by local increases in Ca_o^{2+} owing to cellular death or other mechanisms.

E. Breast Ductal Cells

The CaR is expressed at robust levels in normal breast duct cells, in the duct cells of fibrocystic breast tissue, and in ductal carcinomas of the breast (97). Ca_o^{2+} plays key roles in the breast, not only in its normal physiological function(s) but also in various pathological

states. For instance, Ca_o^{2+} is clearly an important constituent of milk, which has a content of ~200 mg Ca^{2+}/liter (357). A diagnostically important characteristic of breast cancers that can be very useful in their radiological detection during mammographic screening is their tendency to form microcalcifications within ducts (150). Furthermore, in vitro studies have shown that elevating Ca_o^{2+} induces terminal differentiation of normal human breast epithelial cells maintained in culture (289).

There is only limited information available related to the regulation of calcium transport into milk, but the CaR's presence in ductal cells raises the possibility that it could participate in controlling such transport processes. Moreover, metastatic breast cancer has a marked tendency to spread to bone (346). Given the recent demonstration that numerous cells within the bone marrow express the CaR under normal circumstances (204), perhaps the CaR's presence on malignant breast cells contributes to the propensity of these and other CaR-expressing cancer cells to metastasize to bone, in which locally high levels of Ca_o^{2+} are present during bone resorption (412). In addition, we have recently found (J. L. Sanders and E. M. Brown, unpublished data) that high Ca_o^{2+} stimulates the secretion of PTHrP from some breast cancer cell lines in vitro. This high Ca_o^{2+}-stimulated, presumably CaR-mediated, secretion of PTHrP could contribute to the excessive osteolysis caused by breast cancers (107, 166, 167) metastatic to bone by promoting a feedforward mechanism in which release of calcium from the bone stimulated by PTHrP stimulates further PTHrP production, more bone resorption, and so forth. Interrupting this cycle by blocking the activity of the CaR (318) could potentially offer substantial therapeutic benefit in this situation. Therefore, given the importance of Ca_o^{2+} in both the physiology and pathophysiology of the breast, the CaR could play diverse and important roles in these processes.

F. Keratinocytes

Among the tissues uninvolved in systemic Ca_o^{2+} homeostasis that express the CaR are keratinocytes (29). Increases in Ca_o^{2+} are well known to trigger the differentiation of human and mouse keratinocytes in vitro (191, 351). Ca_o^{2+}-evoked differentiation of keratinocytes is accompanied by changes in several intracellular signaling pathways, including accumulation of inositol phosphates (304) and elevations in Ca_i^{2+} resulting from both release of Ca^{2+} from intracellular stores and Ca^{2+} influx through NCC (29). Bikle et al. (29) have recently identified transcripts for the CaR in human keratinocytes, and the differentiating stimulus of a rise in Ca_o^{2+} leads to an increase in the level of CaR mRNA. Therefore, the CaR could mediate the known effects of Ca_o^{2+} on keratinocyte differentiation (29), at least in part, by activating PLC (89, 244, 393) and NCC (471–473), as it does in a number of other cell types.

There are several interesting features of the induction of differentiation of keratinocytes by increases in Ca_o^{2+}. First, only very small increases in Ca_o^{2+}, e.g., from levels less than ~0.05 mM to those greater than 0.1 mM, are needed to induce differentiation. These levels are 10-fold or more lower than those that activate the cloned CaR. Nevertheless, recent studies have shown that the calcimimetic CaR activator, NPS *R*-467, but not its less active stereoisomer, NPS *S*-467, potentiates the actions of Ca_o^{2+} on several aspects of the function of keratinocytes, including increases in Ca_i^{2+} and inositol phosphates and also upregulates the expression of the involucrin and transglutaminase genes (435). Increases in Ca_o^{2+} within the same range also promote the differentiation of other epithelial cells known to express the CaR, including human mammary epithelial cells (289) and chick intestinal goblet cells (31). It will be of interest to determine whether the CaR also mediates those effects.

Oda et al. (327) have recently identified a splice variant of the CaR in keratinocytes whose expression increases as differentiation progresses. This alternatively spliced CaR lacks exon 5 and has an in-frame deletion of 77 amino acids within its extracellular domain. This truncated form of the CaR does not elicit high Ca_o^{2+}-evoked increases in inositol phosphates when transfected in HEK293 cells or keratinocytes. Furthermore, it exerts a dominant negative action on the function of the coexpressed full-length CaR (327). This latter observation provides a plausible explanation for the reduced responsiveness of differentiated keratinocytes to Ca_o^{2+}-induced elevations in Ca_i^{2+} (327). It is at present unknown whether this alternatively spliced version of the CaR is expressed in and serves biological roles in tissues other than the skin.

F. Gastrointestinal System

1. Esophagus

The only study to date of the CaR in the esophagus demonstrated the receptor's presence in the basal cells of the stratified squamous epithelium of the rat esophagus (98). It will be of interest to determine whether the CaR in these cells participates in promoting the differentiation of the esophageal epithelium as it does in the skin or serves other functional roles (29, 327).

2. Stomach

Increases in Ca_o^{2+} stimulate acid secretion by gastric glands and bicarbonate secretion by the gastric surface epithelium (142, 154). These observations suggest that gastric parietal and surface epithelial cells are capable of

sensing changes in Ca_o^{2+}. Cheng et al. (98) have employed RT-PCR to amplify products from RNA isolated from rat forestomach and glandular stomach that were 99% homologous to CaR transcripts expressed in rat kidney. In addition, Northern analysis revealed the presence of CaR transcripts in both the mucosa and muscularis of rat stomach. Immunohistochemistry showed that CaR protein was expressed on mucous-producing surface cells and the acid-secreting parietal cells of the body of the rat stomach (98), although it was not established whether the CaR had predominantly an apical and/or basolateral distribution on these two cell types. A recent study has extended this work to show that the CaR is expressed at the highest level on the basolateral aspect of cultured human gastric surface cells and at lower levels on the apical membrane (396). Activation of the receptor stimulates the proliferation of these cells in vitro. The CaR might participate, therefore, in the known role of these cells in responding to injuries to the epithelial covering of the stomach in vivo. The CaR is also present on gastric epithelial cells of the amphibian, *Necturus* (the so-called mudpuppy) (104). RT-PCR amplified a DNA fragment that exhibited 84% nucleotide identity with the cDNA encoding the rat kidney CaR. Immunohistochemical localization revealed that the CaR was present on the basal surface of mudpuppy gastric surface cells, similar to its localization in human surface cells (104). Electrophysiological studies demonstrated that activating the amphibian CaR with its polycationic agonists or a calcimimetic CaR activator (R-467) (320) decreased basolateral membrane resistance in these cells. These effects were blocked by inhibitors of prostaglandin synthesis. It will be of interest to determine whether in *Necturus* there is a different isoform of CaR and if its signal transduction cascade is predominantly through PLA_2. The degree of homology between the mudpuppy (104) and mammalian CaRs (89), however, is similar to that between the avian (126) and mammalian receptors; therefore, it is likely that all represent the various species homologs of the same ancestral gene.

In addition to its likely role in gastric surface cells, CaR agonists stimulate increases in Ca_i^{2+} in parietal cells within isolated gastric glands and also potentiate the increases in Ca_i^{2+} elicited by histamine (98), indicating that the receptor could mediate the known action of high Ca_o^{2+} to stimulate gastric acid production (142, 154). As in other segments of the gastrointestinal tract, the CaR was also heavily expressed in the enteric nervous system, both in Auerbach's as well as Meissner's plexi (see also sect. XIII*A*), where it could potentially contribute to the control of gastric secretion and motility (98).

In addition to being present in the surface epithelial cells of the stomach and those within the gastric pits, recent studies have localized the CaR in the gastrin-secreting cells of the gastric antrum (374). CaR transcripts and protein were identified using RT-PCR as well as immunocytochemistry and Western blot analysis, respectively, of primary cultures enriched in human gastrin-secreting cells. The CaR expressed in these cells may mediate the long-recognized but poorly understood stimulatory effect of elevated levels of Ca_o^{2+} on gastrin secretion in vitro and in vivo (22, 23, 27). The receptor could also contribute to (e.g., via increases in gastrin secretion) the stimulatory effect of high Ca_o^{2+} on gastric acid secretion (22, 27, 142). Further work is required to prove that it is indeed the CaR that mediates these actions of Ca_o^{2+} and to elucidate the signal transduction pathways through which it acts in this tissue. It is of interest, as discussed in detail in section XIV*B2*, that stimulation of gastric acid secretion produces levels of Ca_o^{2+} within the gastric lumen that are substantially lower (60–75%) than those in the blood. This gradient in Ca_o^{2+} or the absolute level of Ca_o^{2+} on the blood side of the cells, which probably increases owing to the accompanying "extraction" of Ca^{2+} from the gastric juice being elaborated, could conceivably activate CaRs present on their basolateral surfaces and thereby modulate gastric acid secretion (98, 396).

3. Small intestine

Increasing Ca_o^{2+} decreases the proliferation and stimulates the differentiation of intestinal epithelial cells (i.e., goblet cells) in culture (31). How Ca_o^{2+} produces these effects is not known (38, 68). The CaR is expressed throughout the rat small intestine and represents, therefore, a candidate for the known actions of Ca_o^{2+} on various aspects of intestinal function, such as those just noted (85). The use of RT-PCR on RNA isolated from rat duodenal mucosa, duodenal muscularis, jejunum, and ileum amplified a fragment with >99% nucleotide identity to a portion of the rat kidney CaR cDNA corresponding to a region within the receptor's NH_2 terminus (85). In addition, Northern analysis demonstrated 4.1- and 7.5-kb transcripts in each of these tissues. Immunohistochemistry with CaR-specific antisera showed clear basal staining on villus and crypt epithelial cells of the small intestine (with only faint apical staining of the villus cells) (85). Finally, the use of in situ hybridization and immunohistochemistry also demonstrated CaR expression in Auerbach's myenteric plexus of the small intestine, within the submucosa of the duodenum, and in Meissner's plexus and Brunner's glands (85).

What are the functional implications of the CaR within various cell types within the small intestine? Hypercalcemia reduces gastrointestinal motility, whereas hypocalcemia is associated with increased motility (419). In view of the CaR's presence within the enteric nervous system throughout the small and large intestines [as well as in the stomach (98)], it may well be that alterations in systemic levels of Ca_o^{2+} affect gastrointestinal motility in a CaR-mediated fashion. Direct investigation of the ef-

fects of specific CaR activators (320) and antagonists (318) on intestinal motility, coupled with the use of intestinal tissues from homozygous knockout mice for such studies, will be required to address this issue definitively.

Expression of the CaR has also been reported in three adenocarcinoma-derived, intestinal cell lines, which might potentially provide useful model systems for examining the receptor's functional roles in the intestine at the cellular level, viz., T84, HT-29, and Caco-2 (151). The evidence for CaR expression was obtained by RT-PCR using intron-spanning primers located within a portion of the receptor's cDNA encoding its extracellular domain (thereby precluding amplification of similarly sized products from contaminating genomic DNA). Northern analysis using a biotinylated riboprobe generated from the human CaR showed varying levels of CaR expression in the three cell lines (HT-29 > T84 > Caco-2) (151). CaR agonists evoked transient increases of Ca_i^{2+} in HT-29 cells, which were prevented by pretreatment of these cells with a PI-PLC inhibitor or thapsigargin, strongly suggesting that calcium was being mobilized from its intracellular stores via a CaR-mediated, PLC-dependent mechanism (151). Further evidence of the CaR's role in mediating these actions in the three cell lines will, however, require the use of specific CaR activators or antagonists, or transfection of the cells with dominant-negative mutants or antisense constructs.

Given the known actions of other GPCRs on the function of small intestinal epithelial cells, it will also be of interest to address the following issues related to the CaR's potential actions in the small intestine. Does Ca_o^{2+}, acting through the CaR, modulate the secretion of Cl^- stimulated by agents raising cAMP as in T84 cells (24, 224)? Does activating the CaR mimic the action of carbachol to inhibit Cl^- secretion through a mechanism thought to involve stimulation of the MAPK cascade (240)? It will also be of interest to determine whether the receptor affects other secretory processes, such as mucin production (144, 202), or modulates the absorption of Ca^{2+} and/or other nutrients in this portion of the gastrointestinal tract.

4. Colon

In the epithelial cells of the rat colon, there is clear expression of the CaR on both their apical and basal membranes as well as in the enteric nervous system along the entire large intestine (85). The growth and differentiation of colonocytes are exquisitely sensitive to changes in Ca_o^{2+} (31, 68, 168, 457). In cell culture, low levels of Ca_o^{2+} stimulate proliferation, while elevating Ca_o^{2+} inhibits growth and promotes differentiation, as in keratinocytes (191, 351). There is a growing body of circumstantial evidence from epidemiological studies that the incidence of colorectal cancer is inversely correlated with dietary calcium intake (153, 200, 416). It is presently unclear whether this action of Ca_o^{2+} in vivo is indirect, involving, for instance, formation of insoluble salts of calcium with potentially tumorigenic fatty acids and bile salts, or direct (e.g., by inhibiting cellular proliferation of colonocytes) (38, 457).

Kallay et al. (225) recently employed Caco-2 cells as a model system to examine the direct actions of Ca_o^{2+} in vitro on this colon cancer-derived cell line. These cells express the CaR as assessed by RT-PCR and immunohistochemistry performed with a CaR-specific monoclonal antibody. At levels of Ca_o^{2+} of 0.025–0.25 mM, thymidine incorporation into DNA was increased. These low concentrations of Ca_o^{2+} also caused a rapid, PKC-dependent increase in c-*myc* protooncogene expression. When grown on semipermeable supports, to allow addition of medium to either the apical or basolateral surface of the cells, elevating Ca_o^{2+} to 1.8 mM on the apical but not on the basal cell surface blocked the increase in c-*myc* expression (225). This result suggested that activation of the CaR on the apical membrane prevented the increase in c-*myc* expression that was required to induce cellular proliferation. Because the EC_{50} for activation of the CaR in HEK cells (~3.9 mM) (15) and parathyroid cells (~1.0–1.25 mM) (50) by Ca_o^{2+} is so much greater than the level of Ca_o^{2+} required to inhibit growth of Caco-2 cells, it is currently not certain that CaR mediates the inhibitory effect of raising Ca_o^{2+} on colonocyte growth. It will be important to utilize antisense or dominant-negative constructs of the CaR as well as specific CaR activators and antagonists to address this point further.

5. Does the CaR modulate chloride secretion in intestinal crypt cells and other secretory epithelia?

There is now broad recognition that chloride secretion from intestinal crypt cells and other epithelial cells involves a mechanism requiring apical chloride channels, such as the cystic fibrosis transmembrane regulator (CFTR) or a Ca^{2+}-activated chloride channel, together with a basolateral Na^+-K^+-$2Cl^-$ cotransporter, K^+ channels, and the Na^+-K^+-ATPase pump. The presence of the CaR on the basal membrane of intestinal crypt epithelial cells (which may be viewed as an archetypal Cl^--secreting cell) raises the possibility that it could also be present on other Cl^- secretory cells that use the same mechanisms for secretion. To date, however, there are no data available on the CaR's role in regulating Cl^- secretion in crypt or other epithelial cells, although the receptor has recently been shown to stimulate bicarbonate secretion in the acinar cells of the exocrine pancreas and in pancreatic ductal cells (see sect. XIII*H*) (63). Further studies are needed to determine whether the CaR in cells

such as the ductal cells of the pancreatic, biliary, or submandibular glandular cells regulates the secretion of chloride as well.

H. Pancreas

Bruce et al. (63) have recently identified the CaR in the rat pancreas and characterized some of its functional properties. Using a combination of RT-PCR with CaR-specific primers and immunohistochemistry with an anti-CaR antiserum, these workers demonstrated that a CaR-like molecule is expressed in pancreatic acinar cells, epithelial cells of the pancreatic ducts, and in the islets of Langerhans, although the specific cell types in the latter containing the receptor were not identified with certainty. The function of the CaR was assessed in isolated acinar cells and interlobular ducts by measuring the effects of polycationic CaR agonists on Ca_i^{2+} in fura 2-loaded cell preparations. Both cell types responded to addition of extracellular Gd^{3+} or raising Ca_o^{2+} with modest increases in Ca_i^{2+} (63). The effect of activation of the CaR on bicarbonate secretion by isolated ducts was then determined by measuring changes in intracellular pH. Luminal extracellular Gd^{3+} was a potent stimulator of bicarbonate secretion and was equal in efficacy to elevation in intracellular cAMP caused by addition of forskolin. The results of this study suggest that the CaR within the exocrine pancreas senses the level of Ca_o^{2+} in pancreatic juice and could participate in regulating luminal Ca_o^{2+} under basal as well as stimulated conditions so as to mitigate the risk of the formation of calcium carbonate stones owing to excessively high levels of Ca_o^{2+} (63).

The putative functional role(s) of the CaR in pancreatic islets requires further studies. Recent investigations have shown the receptor to be present in the insulin-secreting β-cells, where the CaR could potentially mediate the stimulatory effects of Ca_o^{2+} on insulin secretion that have been shown in normal β-cells (373) and in tumor cells derived from the β-cells (236). Interestingly, however, a recent study showing that normal pancreatic β-cells express the CaR has demonstrated that Ca_o^{2+} can inhibit insulin secretion from these cells under certain conditions (418). Further studies are needed to understand the CaR's normal physiological role, if any, in pancreatic β-cells. The same study also showed that the glucagon-secreting δ-cells of the human islet expressed the CaR (418). It will be of interest in future studies to determine whether the receptor mediates the inhibitory action of Ca_o^{2+} on glucagon secretion that can be demonstrated under specific circumstances (130, 261), and whether it is also present in and regulates the function of other cell types within the islet.

XIV. PHYSIOLOGICAL BASIS FOR LOCAL EXTRACELLULAR CALCIUM SIGNALING

A. Role of Local Levels of Ca_o^{2+} in Systemic Ca_o^{2+} Homeostasis

The homeostatic mechanism illustrated in Figure 6 is remarkable for the precision with which it maintains near constancy of Ca_o^{2+}. From the discussion to this point, it is clear that CaR-expressing, Ca_o^{2+}-sensing cells are key elements within this system, acting as calciostats that can sense and correct small changes in Ca_o^{2+}. Cells participating in systemic mineral ion homeostasis that detect Ca_o^{2+} in some cases presumably respond primarily to systemic levels of Ca_o^{2+}, similar to those measured in the peripheral blood (e.g., parathyroid or C cells). In other cases, however, the levels of Ca_o^{2+} within tissues involved in mineral ion metabolism must differ from that present in blood. For instance, an extreme example is the level of Ca_o^{2+} beneath a resorbing osteoclast, which has been measured to be as high as 8–40 mM (412). Conversely, Ca_o^{2+} would be expected to be lower than its systemic level near sites of active bone formation owing to depletion of calcium ions as a result of their removal from a soluble into an insoluble phase (371, 372). Indeed, blood aspirated from the bone marrow of patients with widespread osteoblastic metastases of prostate cancer to bone can exhibit a value for Ca_o^{2+} that is 20% lower than that present in systemic blood (292). Presumably, exuberant formation of new bone stimulated by the cancer cells in this setting occurs more rapidly than Ca_o^{2+} can be delivered from the circulation.

Local levels of Ca_o^{2+} also likely differ from the "average" level of calcium in blood near sites where calcium ions are being translocated across epithelial interfaces. A good example is the CTAL of the kidney. The level of Ca_o^{2+} within the tubular fluid is similar to or slightly lower than that of the initial glomerular filtrate, which, in turn, is similar to that of ultrafiltrable Ca^{2+} in blood (123, 424). When stimulated by hormones raising cellular cAMP levels in CTAL, the magnitude of the lumen-positive transepithelial potential difference increases, driving passive reabsorption of Na^+, Ca^{2+}, and Mg^{2+} via the paracellular route (123, 183). Furthermore, there is little reabsorption of water in this so-called "diluting segment" of the nephron so that the levels of Ca_o^{2+} to which the basolateral surface of the tubular epithelial cells of the CTAL are exposed may be substantially higher than those in the tubular fluid. Similarly, during the active absorption of calcium ions from the gastrointestinal tract occurring under the influence of vitamin D and after ingesting a Ca^{2+}-containing meal, the level of Ca_o^{2+} within the interstitial fluid at the basolateral side of the intestinal epithelial cells would likely be well above that in the blood. In

fact, interstitial Ca_o^{2+} within the duodenal submucosa has recently been shown to increase nearly twofold when the lumen is perfused with a fluid containing 10 mM Ca_o^{2+}, as noted in section XIII*G* (306). Thus, unlike the Ca_o^{2+}-sensing cells that secrete CT and PTH, Ca_o^{2+}-sensing cells within the effector tissues involved in controlling the movements of calcium ions into or out of the ECF in bone, intestine, and kidney may encounter concentrations of Ca_o^{2+} substantially different from those in blood. Ultimately, these levels of Ca_o^{2+} must be quantified during the responses of the mineral ion homeostatic system to various stresses to understand fully how Ca_o^{2+}-sensing cells participate in systemic Ca_o^{2+} metabolism at both systemic and local levels.

B. Other Microenvironments With Levels of Ca_o^{2+} That Differ From Its Systemic Level

Most tissues that do not participate in systemic Ca_o^{2+} homeostasis presumably do not gain or lose net quantities of calcium in response to changes in the circulating levels of calciotropic hormones that are designed to normalize the systemic level of Ca_o^{2+}. Nevertheless, as is elaborated on in the remainder of this review, it is apparent, on the one hand, that there are diverse microenvironments in which Ca_o^{2+} either differs from its systemic level or changes largely independently of the latter. On the other hand, it is equally clear that numerous cell types express the CaR that are seemingly uninvolved in systemic Ca_o^{2+} homeostasis (e.g., sect. XIII). In some instances, these cells may participate in the regulation of "local" Ca_o^{2+} homeostasis, sensing changes in Ca_o^{2+} within their immediate microenvironments and adjusting the translocation of either ions (e.g., divalent cations) or water so as to adjust the local ionic composition in a physiologically relevant manner. The tubular fluid of the IMCD may represent a good example of such a local homeostatic mechanism. CaRs on the apical membrane of the tubular epithelial cells of the IMCD appear to modulate vasopressin-stimulated water flow so as to set an upper limit to the level to which Ca_o^{2+} is allowed to rise (400, 401), thereby perhaps minimizing the risk of precipitation of calcium-containing salts in the tubular lumen (181).

In other cases, cells may utilize extracellular ionic clues that are characteristic of specific microenvironments to control cellular functions having nothing to do with either systemic or local Ca_o^{2+} homeostasis. In the discussion that follows, we first review circumstances in which local levels of Ca_o^{2+} differ from that in systemic blood and then give several examples of how CaR-mediated Ca_o^{2+} sensing may contribute to the regulation of the local ionic milieu (i.e., control local Ca_o^{2+} homeostasis) or provide information utilized by cells for other, nonhomeostatic purposes. There are a number of instances where local levels of Ca_o^{2+} have been unequivocally shown to differ from corresponding systemic levels, which are categorized in terms of their underlying mechanisms.

1. *Locations where Ca_o^{2+} from the environment contributes to variations in Ca_o^{2+}*

As noted before, there is only intermittent availability of dietary Ca^{2+} to free-living terrestrial organisms. As a consequence, the levels of Ca_o^{2+} and other ions in the lumen of the gastrointestinal tract can vary substantially. For instance, the level of Ca_o^{2+} directly measured in reconstituted, dried milk is ~7 mM (306). Because cells in both the stomach (374) and small intestine express the CaR on their luminal surfaces, these cells will likely experience variations in Ca_o^{2+} that could modulate their functions in physiologically relevant ways. Studies currently ongoing should clarify considerably the importance of this Ca_o^{2+} sensing in the regulation of various aspects of these cells' functions (85, 98, 374).

2. *Changes in local Ca_o^{2+} as a consequence of epithelial ionic transport*

Translocation of calcium ions across epithelial interfaces in the kidney and elsewhere takes place via transcellular [viz., in DCT (148)] and/or paracellular [i.e., in CTAL (123, 181)] pathways. In some cases, as in the proximal tubule of the kidney, ionic transport occurs in such a fashion that the composition of the reabsorbed fluid generally reflects that present in the tubular fluid itself (e.g., with regard to the levels of Na^+, K^+, Ca^{2+}, and Cl^-) (123, 424). In other instances, however, certain ions are selectively reabsorbed, sometimes without accompanying water, thereby modifying substantially the concentrations of Ca_o^{2+} and/or the other ions within the fluid being reabsorbed (and, by extension, those remaining within the tubular lumen). For example, in proximal segments of the nephron, there is less reabsorption of Mg^{2+} than of monovalent ions and Ca^{2+} as noted earlier (123). Consequently, the level of Mg_o^{2+} within the tubular fluid increases progressively until it is ~1.8-fold higher in the thick ascending limb than in the initial glomerular filtrate. In contrast, the level of Ca_o^{2+} rises only modestly in the tubular fluid of the nephron segments proximal to the thick ascending limb. Na^+, K^+, Ca^{2+} , Mg^{2+}, and Cl^- are reabsorbed in the latter segment via both transcellular and paracellular routes without accompanying water (247). Thus the concentrations of both Ca_o^{2+} and Mg_o^{2+} that the CaRs on the basolateral surface of the tubular epithelial cells will experience should be significantly higher than those in either the tubular fluid or systemic ECF. These local changes in Ca_o^{2+} taking place as a result of ionic transport by epithelial cells could provide signals to CaRs that are substantially independent of systemic levels of Ca_o^{2+}. For instance, a reduction in the systemic

level of Ca_o^{2+} will enhance tubular reabsorption of Ca_o^{2+} in the CTAL and presumably be associated with actual increase in the level of Ca_o^{2+} within interstitial fluid on the basolateral side of CTAL cells. An interesting issue that could be relevant to these local changes in Ca_o^{2+} and to Ca_o^{2+}-sensing mechanisms within the immediate microenvironment is the impact of the so-called "unstirred layers" of fluid close to biological membranes, such as at the outer face of the plasma membrane.

The specific ion-transporting properties of a given tissue could generate levels of Ca_o^{2+} differing markedly from those in the systemic ECF. For instance, lactating mothers produce on the order of ~200 mg of calcium daily in each liter of milk, which would result in a concentration of Ca_o^{2+} in milk that is approximately twice that in blood (357). An even more extreme example is afforded by the prostatic fluid, where Ca_o^{2+} reaches 30 mM (436). Ionized levels of Ca_o^{2+} in these fluids are no doubt lower due to the binding of Ca^{2+} to proteins and/or other ions [e.g., to citrate in prostate fluid, thereby lowering the level of ionized calcium below 1.0 mM (384)], but they could still differ substantially from those in systemic ECF and modulate CaRs known to be present, for instance, in the ductal epithelial cells of the breast (97).

Another example of the impact of epithelial solute transport on the level of Ca_o^{2+} within a specific microenvironment occurs within the stomach. The concentration of Ca_o^{2+} within gastric juice is known to vary inversely with the rate of production of gastric fluid during stimulation with gastrin or histamine and is only 30–40% of that present in blood during maximal stimulation (301). Therefore, calcium is, in effect, being extracted from the gastric juice and presumably accumulates in the interstitial fluid at the basolateral surface of the epithelial cells producing it. Direct measurement of Ca_o^{2+} in the interstitital fluid immediately beneath the gastric mucosa will be of interest to determine whether it increases during treatment with agents stimulating acid production sufficiently to provide a CaR-mediated "feed-forward" mechanism that could further enhance the production of gastrin (374) and gastric acid (22, 27, 142) (see also sect. XIII*G*). Such local changes in Ca_o^{2+} could also potentially modulate the activity of CaRs on cells within the enteric nervous system.

3. *Alterations in local Ca_o^{2+} resulting from movement of water without ions*

In contrast to the situation in the thick ascending limb, where Ca^{2+} and Mg^{2+} are transported without accompanying water, the reverse is true in the IMCD (1); that is, reabsorption of water takes place largely without accompanying ions at rates that vary depending on the activity of vasopressin-stimulated aquaporin-2 water channels in the apical membrane. This mechanism enables the kidney to adjust the amount of "free" water that is retained or excreted. As noted earlier, these alterations in the reabsorption of water can be accompanied by changes in Ca_o^{2+} within the tubular fluid in the IMCD that are sufficiently great to promote renal stone formation if there were excessive water reabsorption at the time a Ca^{2+} load is being excreted (400, 401, 436). As noted previously, the CaR's presence on the apical (i.e., luminal) surface of the IMCD tubular epithelial cells affords an example of how this receptor may participate in local Ca_o^{2+} homeostasis. Because elevations in Ca_o^{2+} within the tubular fluid in the IMCD reduce vasopressin-stimulated reabsorption of water, probably through a CaR-mediated mechanism(s), a rise in Ca_o^{2+} in the tubular fluid to an excessively high level can feed back to lower Ca_o^{2+} within the fluid in a homeostatically appropriate manner (183). Interestingly, in contrast to the homeostatic mechanism governing systemic Ca_o^{2+} homeostasis, which adjust the level of Ca_o^{2+} largely by modifying the movements of Ca^{2+} into and out of the ECF (i.e., via intestine, bone, and kidney), in the IMCD the CaR appears to regulate Ca_o^{2+} principally through controlling the movement of water but not Ca^{2+}.

4. *Alterations in local Ca_o^{2+} owing to fluxes of Ca^{2+} between the intra- and extracellular spaces*

A substantial body of evidence documents that Ca_o^{2+} changes appreciably in specific microenvironments owing to alterations in the fluxes of Ca^{2+} between the intra- and extracellular compartments. Substantial changes in Ca_o^{2+} take place in the narrow intercellular spaces of the brain owing to changes in the activity of neurons that are accompanied by cellular uptake of Ca^{2+} through various calcium-permeable channels (e.g., NMDA channels) (9, 186, 271). These alterations in Ca_o^{2+} can be accompanied by changes in the extracellular levels of extracellular Na^+ and extracellular K^+ as a result of influx of Na^+ through voltage-sensitive Na^+ channels and efflux of K^+ though Ca^{2+}-activated and other K^+ channels. A particularly striking example of such changes in the extracellular ionic composition in the ECF of the brain is provided by those occurring during strong electrical stimulation of the cerebellum of the anesthetized rat (323). During electrical stimulation lasting for even a few seconds in this model, Ca_o^{2+} can decrease by up to 90%, whereas extracellular K^+ rises by severalfold. These alterations are rapidly reversible (within a matter of seconds) after termination of the stimulation. Even much milder, more physiologically relevant activation of neurons can be accompanied by easily detectable changes in Ca_o^{2+} that could be sensed by CaRs within the immediate vicinity. For instance, stroking an anesthetized cat's paw with a camel hair brush decreases Ca_o^{2+} by several percent in the ECF within the contralateral primary somatosensory cortex innervating the stroked paw (186). While the magnitude of this alteration

in Ca_o^{2+} may appear small, the CaR on parathyroid cells readily detects reductions in Ca_o^{2+} of this magnitude (e.g., Fig. 7).

We have used modeling to examine the special case of activity-dependent reductions in Ca_o^{2+} within the synaptic cleft (443). A rise in Ca_i^{2+} in the postsynaptic dendritic spines of the hippocampus owing to influx of Ca^{2+} via NMDA channels participates importantly in the induction of LTP (211). Ca^{2+} influx within or close to synaptic clefts also plays key roles in all steps of synaptic transmission, both in the pre- and postsynaptic compartments (32). The synaptic cleft can be visualized as a thin disk of ECF. During stimulation of a synapse at high frequencies, the interval between pulses might potentially be insufficient for Ca_o^{2+} to diffuse in from the periphery of the cleft to replace that lost at the cleft's center owing to activation of postsynaptic, Ca^{2+}-permeable channels. We elaborated a computer model to estimate the alterations in Ca_o^{2+} that might be occurring in the synaptic cleft as a result of the influx of Ca^{2+} into the postsynaptic compartment via iGluRs, combined with subsequent efflux via the calcium pump and Na^+-Ca^{2+} exchanger (443). The levels of Ca_o^{2+} within the cleft were approximated utilizing a compartmental model incorporating fluxes across the postsynaptic membrane combined with radial diffusion in from the edge of the cleft.

Resultant simulations using this model suggested that substantial reductions in Ca_o^{2+} can take place in synaptic clefts attendant on activation of iGluRs, especially at the high stimulation frequencies required to induce LTP. Only minimal, transitory alterations in Ca_o^{2+}, in contrast, were predicted by the model at low frequencies of stimulation. These frequency-dependent changes in Ca_o^{2+} reflect the activity of iGluRs and could potentially modulate presynaptic function through a mechanism involving changes in Ca_o^{2+}, which then serves as a retrograde messenger, if Ca_o^{2+} sensors were resident on the presynaptic membranes. The CaR is known to be expressed on nerve terminals in hippocampus and other areas of the brain (although additional work is needed to define whether it is located pre- and/or postsynaptically), and it could potentially play such a role (87, 388, 392).

In contrast to the reductions in Ca_o^{2+} predicted to take place during the initial phase of electrical stimulation of a synapse by this model, eventual return of the system to its steady state must involve transient increases in Ca_o^{2+} as Ca^{2+} is pumped out of the postsynaptic spine (443). Depending on the "set" of CaRs in the immediate vicinity (e.g., whether they are poised to respond more sensitively to increases or to decreases in Ca_o^{2+}), activity-dependent changes in the function of the CaR, if they exist, might occur primarily during the initial stimulation of the synapse and accompanying Ca_o^{2+} depletion or during the recovery phase, when Ca_o^{2+} rises above its resting level. Furthermore, the overall (e.g., phasic or oscillatory) pattern of changes in Ca_o^{2+} could potentially encode important physiological signals that are decoded by CaRs or other Ca_o^{2+} sensors in the vicinity, as has been suggested to occur intracellularly (5, 34, 94).

Another tissue in which increases in cellular activity produce transient reductions in Ca_o^{2+} is the beating heart (28). Pacing of a frog heart in vitro leads to substantial decreases in Ca_o^{2+} within the interstitial fluid within the heart muscle in this model system (28). Even greater changes in Ca_o^{2+} might occur in this setting within the t-tubular system, where there are narrow infoldings of the plasma membrane into the muscle fibers that ensure close proximity between the ECF within the t tubules and the intracellular sites where Ca^{2+} promotes stimulus-contraction coupling (4).

Two additional examples where Ca_o^{2+} depletion occurs owing to cellular influx of calcium ions from the extracellular fluid are the pancreatic islet and the platelet. During the spontaneous electrical activity of isolated islets investigated in vitro, the periodic initiation of an action potential, which is accompanied by activation of voltage-sensitive Ca^{2+} channels and severalfold increases in Ca_i^{2+}, causes substantial (~0.5 mM) reciprocal decrements in Ca_o^{2+} (347). With this electrical activity ceases, both Ca_i^{2+} and Ca_o^{2+} returned to their basal levels. In addition, when platelets aggregate in vivo, there are substantial (~80%) reductions in Ca_o^{2+}, presumably owing to influx of Ca_o^{2+} into the platelets, which is rapidly reversible when the platelets disperse after addition of an agent inhibiting their aggregation (334).

In contrast to the situations just described, in which reductions in Ca_o^{2+} are initiated by cellular uptake of Ca^{2+}, activation of cells by Ca^{2+}-mobilizing hormones binding to their respective receptors (e.g., those coupled to activation of PI-PLC), can produce an initial rise rather than fall in Ca_o^{2+} following cellular activation. Intracellular Ca^{2+} stores can comprise total cellular calcium concentrations of several millimoles per kilogram (356) (smooth muscle cells can have ~8 mmol/kg, half the calcium content per wet weight of mineralized bone, Ref. 384). Thus extrusion of Ca^{2+} mobilized from these stores by inositol trisphosphate via the plasma membrane Ca^{2+} pump could potentially produce substantial increases in the level of Ca_o^{2+} within narrow intercellular spaces in vivo (72, 75, 199, 431, 468). Subsequent refilling of these cellular stores would then presumably result in transient lowering of Ca_o^{2+} after removal of the Ca^{2+}-mobilizing hormone. Again, either the absolute change in Ca_o^{2+} or the pattern of the change might provide important information that could be decoded by CaRs in the immediate environment. Thus the initiation of Ca_i^{2+} signaling may be accompanied by an obligate activation of Ca_o^{2+} signaling given the impact of changes in Ca_i^{2+} dynamics on Ca_o^{2+} in many cell types and the CaR's wide distribution.

The level of Ca_o^{2+} is ~10,000-fold greater than that of

Ca_i^{2+}. It may be surprising, therefore, that changes in cellular activity, such as those just described, which usually increase the Ca_i^{2+} by ~10-fold or less (300, 350), are able to significantly decrease or increase Ca_o^{2+} when there is cellular Ca^{2+} influx or efflux, respectively. The answer to this apparent paradox lies in two factors: *1*) the restricted spaces in the ECF of intact tissues, which, therefore, contain only limited amounts of Ca_o^{2+} and *2*) the magnitudes of the influx or efflux of Ca^{2+} during cellular activation or recovery, which are much greater than the associated changes in Ca_i^{2+} owing to the presence of intracellular Ca^{2+} buffers. It is also probable that there is substantial heterogeneity in terms of the magnitudes and locations of the changes in Ca_o^{2+} that may occur over the outside of the plasma membranes of individual cells, depending on the locations of the sites of Ca^{2+} influx and efflux as well as of the CaR itself in cells expressing this receptor.

One specific microenvironment of potential interest in this regard is that within caveolae (see sect. VII*C*) (8, 268, 341). Recent studies suggest that caveolae can serve as "message centers" for the cell, since they contain important components involved in signal transduction, such as GPCRs and tyrosine-coupled receptors, G proteins, PKC isoforms, the plasma membrane calcium pump (Ca^{2+}-ATPase), and inositol trisphosphate-regulated channels (8, 268, 341). On the order of 80% of the CaR protein expressed in bovine parathyroid cells is located within caveolae (245). Thus, depending on the relative densities of influx versus efflux pathways for calcium ions within the caveolae, there could potentially be large local fluxes of Ca^{2+}. For instance, consider the hypothetical case in which the CaR in parathyroid cells resides in caveolae containing the Ca^{2+}-ATPase, but calcium influx pathways stimulated by the CaR are elsewhere in the plasma membrane. Activation of the receptor by increases in Ca_o^{2+} would elevate Ca_i^{2+} through both cellular mobilization and influx of calcium ions. The latter would then be pumped out of the cell in the immediate vicinity of the receptor within caveolae and could potentially generate a feed-forward activation of the receptor by elevating the local level of Ca_o^{2+} above that present in the general ECF.

The distribution of Ca^{2+} buffers within the cytosol will likely also alter the patterns of changes in both Ca_i^{2+} and Ca_o^{2+} attendant on cellular activation, since a larger influx of Ca_o^{2+} would be required to bring about a given change in Ca_i^{2+} if influx takes place where there is a locally high concentration of intracellular calcium buffer(s). Thus the presence of Ca_i^{2+} buffers (i.e., calbindins) in CaR-expressing cells, such as parathyroid cells (58, 64), intestinal cells (85, 453), and cells of the DCT (105, 379) could modify local alterations in Ca_o^{2+} occurring in association with changes in Ca_i^{2+}, including those produced by activating the CaR.

The large amounts of Ca^{2+} within intracellular stores can also serve as a reservoir for Ca^{2+} that can raise local levels of Ca_o^{2+} in the setting of inflammation and cell death. Menkin (294) showed over 40 years ago that the fluid within exudative abscesses (e.g., containing many inflammatory cells) induced in dogs by subcutaneous injection of irritants could contain levels of Ca_o^{2+} threefold higher than those measured simultaneously in blood. In contrast, transudative fluid accumulations (e.g., those that are devoid of inflammatory cells) have levels of Ca_o^{2+} similar to those in the blood. It is likely that calcium released by dying inflammatory cells contributed to the high levels of Ca_o^{2+} within the fluid in the exudative abscesses, since the calcium content of most cells is severalfold higher than that in the systemic ECF (384).

Additional examples of fluids in which increases in Ca_o^{2+} have been documented related to inflammation include the fluid from inflamed gingiva (gums) (234, 235) and peritoneal fluid isolated from patients being subjected to peritoneal dialysis for renal insufficiency who develop peritonitis (266). Because elevated levels of Ca_o^{2+} promote the chemotaxis of monocytes (421) and stimulate the formation of multinucleated giant cells from pulmonary alveolar macrophages (215), the elevated levels of Ca_o^{2+} within inflammatory fluids may serve as a signal promoting the influx and activation of macrophages (see also sect. XIII*D*).

5. *Local changes in Ca_o^{2+} resulting from movements of Ca^{2+} into and out of extracellular reservoirs*

Translocation of calcium ions into or out of bone provides an instructive example of how Ca^{2+} fluxes into or out of extracellular reservoirs that contain large quantities of this ion can perturb local (and also systemic) levels of Ca_o^{2+}. As noted before, Ca_o^{2+} underneath a resorbing osteoclast can reach levels as high as 40 mM (412). Although the impact of releasing this resorbed Ca^{2+} into the osteoclast's immediate microenvironment on the local level of Ca_o^{2+} is unknown, it is probable that the latter would rise substantially. Indeed, when there is uncontrolled release of skeletal Ca_o^{2+} from bone, as with skeletal metastases of breast cancer and certain other malignancies, even systemic levels of Ca_o^{2+} can increase to supranormal levels (419). Ca_o^{2+} within the bony microenvironment would presumably be even higher in such circumstances.

In addition to serving as a source of calcium ions (e.g., during resorption of bone), the skeleton can also serve as a "sink" for Ca_o^{2+}. For instance, extensive osteoblastic metastases of prostate cancer to bone can cause systemic hypocalcemia, presumably because exuberant bone formation caused by tumor-derived products (164) outstrips the Ca_o^{2+} homeostatic system's capacity to pro-

vide additional Ca^{2+} via renal conservation, intestinal absorption, and bone resorption (419).

C. Physiological Relevance of Local Ca_o^{2+} Sensing and Ca_o^{2+} Signaling

Therefore, the substantial number of cell types expressing the CaR that are seemingly uninvolved in systemic Ca_o^{2+} metabolism may engage in Ca_o^{2+} sensing in response to Ca_o^{2+} signals that arise largely independent of the homeostatic processes that maintain near constancy of Ca_o^{2+} in the blood (52); that is, local changes in Ca_o^{2+} owing to alterations in cellular activity, ion transport, or the other processes just described may elicit cellular responses by CaR-expressing cells via modes of communication analogous to paracrine or autocrine signaling by more classical hormones or cytokines. In some cases, Ca_o^{2+}-induced activation of the receptor may serve to restore local ionic homeostasis, as in the tubular fluid of the IMCD of the kidney. In other cases, there are tantalizing hints of previously unappreciated roles for Ca_o^{2+} sensing and Ca_o^{2+} signaling at the local level. The chemotactic responses of osteoblasts and their precursors to sites of high Ca_o^{2+} may serve as an important component of the mechanism through which the osteoclastic "injury" to bone is "healed" by new bone formation. As an extension of this concept, the chemotactic response of monocytes to Ca_o^{2+} and the presence of high local levels of Ca_o^{2+} within inflammatory fluids may suggest a broader role for Ca_o^{2+} as a "chemokine" and as a more general inflammatory signal. Furthermore, it is likely that not only Ca_o^{2+} but other endogenous CaR activators and modulators will contribute to regulating the activity of the CaR in local microenvironments, including not only other polycationic agonists, such as Mg_o^{2+}, spermine, Aβ peptides, and perhaps other endogenous polycations, but also ionic strength. Therefore, the CaR will likely integrate information encoded by several different local environmental factors that will ultimately determine the functional impact of the receptor on a given cell.

XV. SUMMARY

The cloning of the G protein-coupled CaR has provided an actual or potential molecular mechanism mediating many of the known effects of Ca_o^{2+} on the cells and tissues that maintain systemic Ca_o^{2+} homeostasis, particularly parathyroid and kidney. In addition to affording useful molecular tools for documenting the presence of CaR mRNA and protein in these tissues, the cloned CaR has permitted the identification of human diseases that are the consequences of inactivating or activating mutations of this receptor as well as to generate mice with knockout of the CaR gene. The characteristic abnormalities in the functions of the parathyroids and kidneys of these patients and in CaR knockout mice have provided the means to dissect the physiological roles of the CaR in mineral ion metabolism. Much remains to be learned, however, about how the CaR regulates other tissues involved in systemic homeostasis, such as bone and intestine, including the role(s), if any, of additional Ca_o^{2+}-sensing receptors/sensors that have yet to be characterized at a molecular level. Moreover, although these human diseases and mouse models will afford useful tools for further investigating the receptor's roles in these latter tissues, the development of potent and specific CaR antagonists, tools that are only just beginning to be developed (318), would be extremely useful in this regard.

In addition, much remains to be learned about the CaR's functions in tissues that are not directly involved in systemic mineral ion homeostasis, where the receptor probably serves numerous additional roles, some related to local intra- and extracellular regulation of ions and others unrelated to either systemic or local ionic homeostasis. In any event, the CaR and perhaps other receptors/sensors for calcium or other extracellular ions will likely be versatile regulators of a wide variety of cellular functions (52, 457) and represent potentially important therapeutic targets.

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