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Parathyroid Hormone Secretion and Action: Evidence for Discrete Receptors for the Carboxyl-Terminal Region and Related Biological Actions of Carboxyl-Terminal Ligands

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PTH is a major systemic regulator of the concentrations of calcium, phosphate, and active vitamin D metabolites in blood and of cellular activity in bone. Intermittently administered PTH and amino-terminal PTH peptide fragments or analogs also augment bone mass and currently are being introduced into clinical practice as therapies for osteoporosis. The amino-terminal region of PTH is known to be both necessary and sufficient for full activity at PTH/PTHrP receptors (PTH1Rs), which mediate the classical biological actions of the hormone. It is well known that multiple carboxyl-terminal fragments of PTH are present in blood, where they comprise the major form(s) of circulating hormone, but these fragments have long been regarded as inert by-products of PTH metabolism because they neither bind to nor activate PTH1Rs. New *in vitro* and *in vivo* evidence, together with older observations extending over the past 20 yr, now points strongly to the existence of novel large carboxyl-terminal PTH fragments in blood and to receptors for these fragments that appear to mediate unique biological actions in bone. This review traces the development of this field in the context of the evolution of our understanding of the "classical" receptor for amino-terminal PTH and the now convincing evidence for these receptors for carboxyl-terminal PTH. The review summarizes current knowledge of the structure, secretion, and metabolism of PTH and its circulating fragments, details available information concerning the pharmacology and actions of carboxyl-terminal PTH receptors, and frames their likely biological and clinical significance. It seems likely that physiological parathyroid regulation of calcium and bone metabolism may involve receptors for circulating carboxy-terminal PTH ligands as well as the action of amino-terminal determinants within the PTH molecule on the classical PTH1R. (*Endocrine Reviews* 26: 78–113, 2005)

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Abbreviations: b, Bovine; CPTH, C-terminal PTH peptide(s); CPTHR, CPTH receptor; C-terminal; carboxyl-terminal; GPCR, G protein-coupled receptor; h, human; NHERF, sodium/hydrogen exchanger regulatory factor; NMR, nuclear magnetic resonance; N-terminal, amino-terminal; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; p, porcine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; proPTH, prohormone; PTH1R, PTH/PTHrP receptor; PTH2R, PTH2 receptor; TIP39, tuberoinfundibular peptide of 39 residues; TMD, transmembrane domain; zPTH3R, type-3 zebrafish PTH receptor.

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I. Introduction

DETERMINATION OF THE amino acid sequence of the PTH molecule in 1970 in the bovine (1) and shortly afterward in other species (2–4) was followed closely by the finding that the major biological activities of PTH are subserved by the amino (N)-terminal 34 residues of the hormone molecule (5–7). Indeed, by the 1980s, most physiological studies of PTH action were carried out using commercially available N-terminal fragments of PTH, usually bovine or human (h)PTH(1–34) rather than intact 84-residue hormone. During the last three decades, it has been the prevailing view that the PTH residues located beyond position 34 were largely irrelevant. This concept was solidified by the cloning of the receptor for N-terminal PTH and PTHrP in 1991 (8, 9) and related research leading to the conclusion that all of the major biological activities of PTH are mediated by binding of N-terminal hormone residues within the (1–34) region to this receptor (5, 10). Over the same three decades, however, it has been realized that carboxyl (C)-terminal PTH fragments are present in the circulation in large amounts. These fragments were presumed to be biologically inactive, because they have no activity at the PTH/PTHrP receptor (PTH1R). Considerable evidence now has accumulated, however, for the presence in kidney and bone of distinct receptors specific for C-terminal sequences within PTH. Very recently, convincing evidence has emerged for biological actions related to these C-terminal PTH (CPTH) receptors (CPTHRs), particularly in the regulation of bone resorption and the serum calcium concentration. Available data from experiments in rats, dogs, cows, and humans indicate that a variety of C-terminal fragments of PTH, derived via both direct secretion from the parathyroid glands and postsecretory proteolysis of PTH, normally circulate in blood at concentrations severalfold higher than that of intact PTH, and at much higher levels in renal failure. Furthermore, the concentrations of these fragments in plasma are regulated by the level of serum calcium, which controls the secretion of C-terminal PTH fragments from the parathyroid glands and also may regulate cleavage of intact hormone to fragments in the liver. This review provides a critical summary of the currently available evidence for the existence and nature of biologically relevant C-terminal receptors for PTH and the regulated presence in the circulation of the ligands for these receptors, PTH, and C-terminal PTH fragments.

II. Structure of PTH

Mammalian PTH is an 84-amino acid single-chain polypeptide, the primary sequence of which was established first by chemical analysis of highly purified bovine hormone (1, 11) and subsequently confirmed by isolation of complementary and genomic DNAs from several different species (2–4, 12–15) (Fig. 1). The intact, secreted form of the hormone is generated by cleavage of a prohormone (proPTH) containing a 6-amino acid N-terminal extension. proPTH, in turn, is produced by proteolysis of a larger, short-lived precursor, preproPTH, which is the initial translation product (16). PreproPTH incorporates an additional 25-residue N-terminal signal sequence, rich in hydrophobic amino acids, that, together with the 6-residue "pro" sequence, is necessary for efficient transport into the endoplasmic reticulum and subsequent cleavage to the mature hormonal form (17–20). The C-terminal portion of PTH also is needed for efficient processing of the hormone. Thus, in cells transfected with cDNA encoding preproPTH(1–40) or preproPTH(1–52), the proPTH forms were not cleaved but, instead, were degraded intracellularly (21). These observations have suggested one possible role for the C terminus of PTH, *i.e.*, to ensure high-fidelity processing and effective transport of the hormone through the parathyroid secretory apparatus.

The primary amino acid sequence of PTH is highly conserved among mammalian species (Fig. 1). The homology is strongest in the N terminus of the molecule, where 32 of the first 38 residues are identical and even those differences that do occur are relatively conservative. The greatest evolutionary variation is evident in the middle region of the hormone, between residues 39 and 52, whereas several stretches of high homology can be found in the C-terminal region, *i.e.*, PTH(53–84). For example, PTH(53–61) shows 100% identity among mammals except for conservative exchanges of acidic residues (Asp for Glu, or *vice versa*) at positions 56 and 61 in the rodent sequences. Several residues are conserved also in

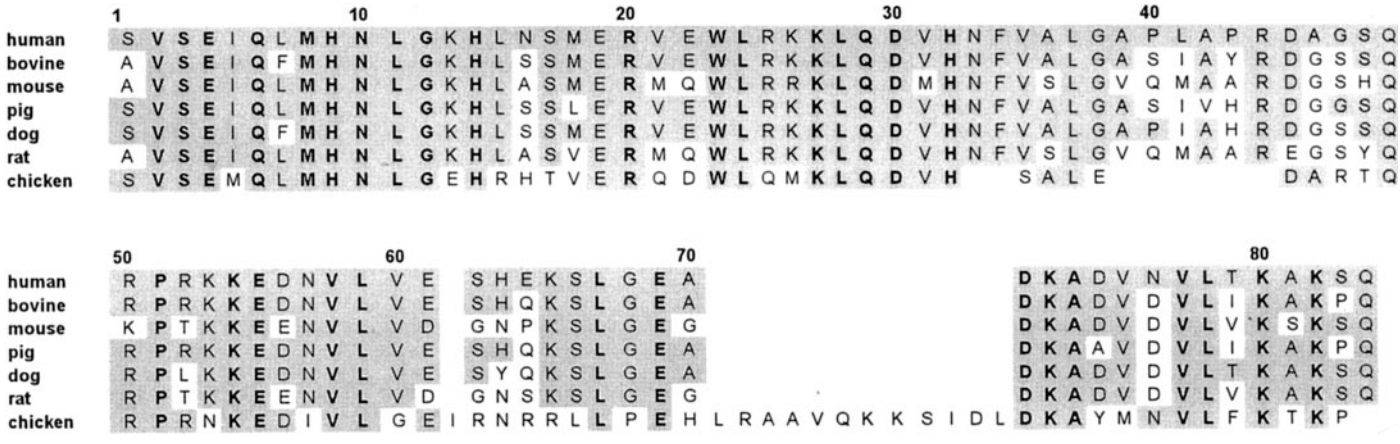

FIG. 1. Amino acid sequences of intact PTH from several mammalian species. Amino acids encoded by PTH cDNAs are denoted by the *single-letter abbreviation* for each of the indicated species. *Shading* indicates residues in hPTH that are conserved in other species, and those residues conserved in all species shown are indicated in *bold*.

the chicken and zebrafish sequences. Furthermore, the 14-residue sequence PTH(65–78) shows variation at only three positions among mammals, and residues Lys^{80}, Lys^{82}, and Gln^{84} are invariant as well. The extensive sequence conservation at the PTH N terminus fits well with abundant evidence that this region of the molecule is both necessary and sufficient for full activation of the classical PTH1R (see *Section III*), whereas the high homology in portions of the C terminus of the hormone had remained enigmatic before the emergence of more recent evidence of the existence of CPTH-specific receptors.

Understanding of the structure of PTH, either alone in solution or, perhaps more importantly, in direct association with its receptor(s), remains incomplete. Early studies using dark field electron microscopy or computational schemes suggested that PTH in aqueous solution consists of two (N- and C-terminal) linked globular domains (22, 23), whereas nuclear magnetic resonance (NMR) and circular dichroism analyses revealed no evidence of extensive secondary structure (24, 25). Considerable effort has been focused on the structures of peptides comprising the N-terminal region of PTH required for PTH1R activation. Thus, numerous solution-phase NMR studies support the presence of two α-helical domains, especially within the regions PTH(3–11) and PTH(21–30), that are joined by a more flexible "hinge" region, a structure permissive of a large number of overall hormone conformations in solution (24, 26–34). A similar secondary structure, involving N- and C-terminal α-helices, was shown for hPTHrP(1–34) in solution by two-dimensional NMR (35, 36). X-ray crystallography of hPTH(1–34), however, predicted the presence of a single linear α-helix extending from Ser^3 to Asn^{33} (37). Analysis of the impact of chemical substitutions that constrain peptide confirmation is also most consistent with the concept that PTH(1–19) binds to the PTH1R as an extended α-helix (38–40). Solution phase two- and three-dimensional NMR analysis of the structure of prolylhPTH(1–84) showed no evidence of secondary structure unless the solvent hydrophobicity was increased by addition of trifluoroethanol (up to 70%) (41). Under these conditions, extensive α-helicity was detected between Ser^3 and Gly^{38}, and this was strongest between Met^{18} and Gln^{29}. Interestingly, the PTH(39–53) region, least conserved at the level of primary sequence (Fig. 1), appeared devoid of secondary structure, whereas evidence of limited structure, *i.e.*, turns and short helices, was found within the C-terminal hPTH(54–84) region. Furthermore, nuclear Overhauser effect analysis confirmed evidence of secondary structure in regions 3–10, 17–27, 30–37, and 57–62, but not within region 40–52.

It is important to emphasize that these biophysical measurements have been performed under experimental conditions with uncertain analogy to the local environment in which the ligand-receptor interaction actually takes place, *i.e.*, at the interface between the extracellular fluid compartment and the cell surface. Given this proviso, one can conclude from available data that regions of PTH, especially those most highly conserved genetically, exhibit some evidence of ordered structure(s) that may be relevant to receptor interaction. More definitive information must await further technical advances, including the possible crystal structure of the active hormone/receptor complex.

III. Classical Actions of PTH and the PTH/PTHrP Receptor

A. Structural basis of PTH signaling and action

The classical actions of PTH, including phosphaturia via a direct renal action (42–44) and elevation of blood calcium (45) via a combined effect of increased osteoclastic bone resorption (46–48), renal tubular calcium reabsorption (49), and renal synthesis of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (50, 51), had been well established. These actions were clearly recognized, for the most part, before the structure of the hormone was known, through careful observations of the responses to parathyroidectomy, parathyroid transplantation, and administration of crude parathyroid extracts (52) to experimental animals or to *ex vivo* organ cultures (53–55). Other actions were noted as well, such as proliferative effects upon blood and liver cells (56–58), but the main thrust of ongoing investigation focused on those biological responses seemingly most directly relevant to maintenance of bone and mineral ion homeostasis. The recognition by Aurbach and colleagues that cAMP is involved as a second messenger in PTH action in bone and kidney, both *in vivo* and *in vitro* (59–65), was a major advance that led to the development of sensitive *in vitro* bioassays and was followed quickly by the isolation, sequencing, and characterization of purified PTH from several species (1, 11, 66–68) and subsequently, the molecular cloning of the corresponding cDNAs (2, 12).

Although the natural hormone is 84 amino acids in length, it was found that a synthetic N-terminal fragment, bovine (b)PTH(1–34), could reproduce the major biological actions attributed to full-length bPTH, including activation of adenylyl cyclase in bone and kidney cells, increased urinary excretion of cAMP and phosphate in rats, and elevation of blood calcium in rats, dogs, and chicks (7, 69–73). Moreover, it was found that synthetic carboxyl fragments such as PTH(44–68), PTH(53–84), and PTH(39–84) did not compete for binding with PTH(1–34) radioligands, nor did they activate adenylyl cyclase in renal membranes or bone cells (74–76). These observations, together with the practical difficulties at that time in reliably producing large quantities of chemically pure PTH(1–84), led to widespread use of synthetic PTH(1–34) as a surrogate for intact PTH in investigations of hormone action *in vitro* and *in vivo*. Detailed structure-function analysis of the PTH(1–34) ligand demonstrated the importance of its extreme N terminus (especially residues 1 and 2) for activation of adenylyl cyclase (7, 77–79) and of its C terminus (*i.e.*, residues 15–34) for high-affinity receptor binding (80–84). Other binding determinants must exist within the N terminus of PTH(1–34), however, because N-truncated peptides such as PTH(3–34) and PTH(7–34) bind with affinities considerably lower than that of PTH(1–34) (74, 80, 85).

The abolition of bioactivity that accompanied progressive N-terminal truncation of PTH suggested a strategy for developing effective PTH antagonists, but initial efforts based

on the use of PTH(3–34) analogs were thwarted when residual agonism, not readily apparent during *in vitro* analyses, was detected *in vivo* (86, 87). Additional truncation to analogs lacking up to six N-terminal amino acids allowed effective inhibition *in vivo*, as with [Tyr^{34}]hPTH (7–34)NH_2, although potency was limited by low binding affinity (88). Subsequent introduction of amino acid substitutions found to enhance binding of such shortened analogs has led to design of even more effective antagonists, such as [Nle^8, D-$Trp^{12,18}$, Tyr^{34}]bPTH(7–34)NH_2, [Leu^{11}, D-Trp^{12}]PTHrP(7–34)NH_2, and [Ile^5, Leu^{11}, D-Trp^{12}, Trp^{23}]PTHrP(5–36)NH_2 (77, 89, 90).

Use of cAMP generation as the exclusive measure of the intracellular action of PTH subsequently became untenable when studies of both bone- and renal-derived cells and tissues demonstrated that PTH(1–34), as well as PTH(1–84), could activate other signal transduction pathways, independently of adenylyl cyclase, including those involving phospholipase C (PLC), protein kinase C (PKC)(s), cytosolic free calcium (Ca^{2+}), phospholipase D, and phospholipase A_2 (91–110). PTH also regulates MAPKs, including p42/p44 ERKs, p38 and c-Jun N-terminal kinase subtypes, although the direction of this regulation and its mediation by more proximal effectors such as cAMP/PKA and PKC, especially in the case of p42/p44 ERKs, appears to depend on cell type and the concentration of PTH (111–119).

Such extensive signaling diversity initially raised the possibility that PTH might interact with more than one type of receptor in these target tissues, although subsequent studies with cloned PTH receptors (see *Section III.B*) showed that such multiple signaling, at least for adenylyl cyclase, PLC, cytosolic Ca^{2+}, and PKC, can be mediated by a single receptor species. Of particular interest were observations that these cAMP-independent responses, notably PKC activation and elevation of cytosolic Ca^{2+}, could be induced by N-truncated PTH fragments or analogs that were unable to effectively stimulate adenylyl cyclase (96, 103–105, 120–123). In particular, a short sequence comprising residues 29–32 of hPTH was shown to be both necessary and sufficient for activation of membrane-associated PKC(s) in osteoblastic cells, whereas the fragment hPTH(1–31), lacking this domain, could not elicit this PKC response (122, 123). Such observations are of interest in the context of the present review for at least two reasons. First, they raise the possibility that biological effects reported for certain C-terminal PTH fragments, devoid of cAMP-stimulating activity but long enough to include the PTH(29–32) domain (see *Section III.B*), might involve activation of PKC via classical PTH1Rs, even if such fragments cannot be shown to compete effectively with N-terminal PTH radioligands for binding to target cells. Second, they could provide a mechanism to explain cAMP-independent biological actions (mitogenesis, regulation of creatine kinase, regulation of other genes) reported for certain midregional PTH fragments, such as PTH(25–39), PTH(28–47), PTH(28–48), and PTH(29–47) (124–131). That these latter actions may reflect interaction of these midregional peptides with PTH1Rs is further supported by the observation that excess PTH(28–48) inhibits the cAMP response to PTH(1–84) (125) and that the PTH(28–42) and PTH(28–48) fragments can activate PKC in CHO cells expressing transfected PTH1R cDNA (132). On the other hand, the anabolic action of intermittently administered PTH via the PTH1R is not seen with intermittent administration of hPTH(28–48) (133). Thus, the *in vitro* mitogenic activity observed in response to this midregion fragment is not linked to stimulation of bone formation *in vivo*.

B. Identification, cloning, and signaling properties of the PTH/PTHrP receptor

The discovery that PTH elicits activation of adenylyl cyclase and production of cAMP predicted that the responsible receptor would be a member of the G protein-coupled receptor (GPCR) family. This concept was further supported by evidence that PTH action could be modulated by guanyl nucleotides (activation of adenylyl cyclase or PLC) or by pertussis toxin (99, 134–138). Direct radioligand binding analysis of the interaction of PTH with membranes or intact cells of skeletal or renal origin demonstrated saturable binding with an affinity constant in the low nanomolar range (74, 75, 137, 139–145), and chemical cross-linking studies indicated that the PTH receptor was likely to be a 60- to 80-kDa membrane glycoprotein (146–152). After the discovery of PTHrP as the cause of the humoral hypercalcemia of malignancy syndrome, the recognition of the high sequence homology of its N terminus with that of PTH, and the demonstration that the biological actions of N-terminal fragments of PTH and PTHrP were equivalent in many different bioassays (153, 154), it was shown that both PTH and PTHrP N-terminal fragments bind to the same receptor sites in kidney and bone cells (155–157).

cDNA encoding the PTH1R was successfully isolated in 1991 by using photoemulsion autoradiography to screen for binding of ^{125}I-hPTHrP(1–36) radioligand by COS-7 cells that were transfected with pcDNA1 plasmid pools from an opossum kidney cell cDNA library (8). Subsequent comparison of cDNAs encoding the opossum, rat, human, and porcine PTH1Rs (8, 9, 158, 159) demonstrated that these specify highly homologous (80–95% amino acid identity) single-chain polypeptides approximately 590 amino acids in length, each featuring an extended N-terminal extracellular domain, the anticipated seven hydrophobic helical transmembrane domains (TMDs), and an intracellular cytoplasmic "tail" containing a number of serine residues that undergo phosphorylation upon agonist interaction (160–163). The N-terminal domain is glycosylated at four asparagine residues clustered near the junction with the first TMD (164), and includes three disulfide bonds involving six highly conserved cysteines (165). The PTH1R is activated equivalently by intact and N-terminal PTH and PTHrP peptides and, like other members of the class II family of GPCRs (including secretin, calcitonin, vasoactive intestinal peptide, glucagon-like peptide-1, GHRH, corticotropin-releasing factor, and glucagon, among others), it is capable of coupling to several different G proteins, thereby activating multiple signaling pathways concurrently, including adenylyl cyclase/cAMP, PLC, cytoplasmic Ca^{2+}, and PKC, when expressed in heterologous cell systems (9, 159, 166–170). The intracellular tail of the receptor seems also to be important for coupling to a pertussis toxin-sensitive Gi protein that inhibits adenylyl cyclase (171). The receptor tail recently was also shown to include a highly

conserved PDZ interaction domain required for binding to the sodium/hydrogen exchanger regulator factor (NHERF) family of adapter/regulatory proteins, which may govern the balance between the PLC of the receptor *vs.* adenylyl cyclase signaling, at least in some cells (172).

The PTH1R is highly expressed in bone and kidney, but is found also in a variety of tissues not regarded as classical PTH target tissues (173, 174). This likely reflects the widespread local paracrine role of PTHrP postulated in tissues such as breast, skin, heart, blood vessels, pancreas, and others (154, 174). Ablation of the PTH1R gene in mice [and inactivating mutations in humans (175)] results in neonatal lethality and a severe defect in endochondral bone formation characterized by impaired proliferation and accelerated chondrocyte maturation and mineralization (176). Such mice have been "rescued" via chondrocyte-specific expression of constitutively active PTH1Rs (177), but these animals display other abnormalities in tooth development and bone, and detailed analysis of mineral ion homeostasis has not yet been possible. Because a phenotype similar to that of the receptor-null animals results from PTHrP gene ablation (178), the predominant endochondral defect likely reflects interruption of the critical local paracrine role of PTHrP in the growth plate (179). Ablation of the PTH gene (180, 181) does lead to hypocalcemia and hyperphosphatemia, whereas activating mutations in the PTH1R cause hypercalcemia and hypophosphatemia (182, 183), confirming the primary role of PTH *per se*, and of the PTH1R, in maintaining normal mineral ion homeostasis. Mice lacking the gene for PTH also exhibit abnormalities in mineralization and formation of primary spongiosa of long bones, which are not seen in PTHrP-null animals and are presumed to reflect loss of PTH-specific actions in bone, although the receptors involved have not been defined (180, 181).

The manner in which the PTH ligand interacts with the PTH1R has been deduced from an extensive series of studies by several groups involving mutagenesis of both the receptor and the ligand, use of hybrid receptors and ligands, and direct chemical cross-linking of ligands bearing photoreactive groups at specific locations within the peptide chain (84, 184–194). These analyses indicate that interaction of the PTH(1–34) ligand with the PTH1R involves high-affinity binding of the C terminus of the ligand with portions of the receptor's extracellular N-terminal domain and the extracellular loops that connect the TMDs. The N terminus of the ligand then interacts with the TMD domains to catalyze the G protein activation(s) required for signal transduction (195, 196). The critical role in receptor activation of the juxtamembrane ("J") domain, comprising the seven TMDs and their connecting intra- and extracellular loops, is highlighted by the fact that the several mutations identified as causing constitutive (*i.e.*, ligand-independent) receptor activation (Jansen's metaphyseal chondrodysplasia) are located within the J domain (182). Moreover, N-terminal PTH peptides as short as PTH(1–14) or PTH(1–11) that incorporate substitutions designed to promote α-helical structure, unlike PTH(1–34), can activate truncated receptors lacking the N-terminal extracellular domain as effectively as wild-type receptors (38, 197).

Studies of the signaling properties of clonal PTH1Rs expressed in heterologous cell lines, such as LLC-PK1, COS-7, or HEK 293 kidney cells or CHO cells, have shown that this single receptor type can mediate activation of adenylyl cyclase, PLC, phospholipase D, PKC, and MAPK and can increase the concentration of cytoplasmic Ca^{2+} (9, 166, 169, 198–201). Thus, it is not necessary to invoke the existence of other types of PTH receptors to explain the diversity of signaling events that N-terminal fragments of PTH or PTHrP can elicit in various target cells, although this possibility has not been excluded completely. Mutational analysis has indicated that different G proteins likely do not interact with the PTH1R identically. For example, a clustered mutation (EKKY→DSEL) in the second intracellular loop of the rat receptor completely abrogates PLC signaling without affecting adenylyl cyclase activation (202), and mice expressing only this mutant receptor display subtle developmental defects in endochondral bone formation (203). Although it is clear that an intact N terminus of PTH or PTHrP is needed for effective adenylyl cyclase activation via PTH1Rs (76, 132, 169, 204, 205), this does not appear to be true for activation of PKC. Thus, as noted earlier, various N-truncated PTH peptides have been shown to activate PKC(s) in cells expressing endogenous or transfected recombinant PTH1Rs (96, 103–105, 120–123, 132, 206). At the same time, studies of cells stably expressing the transfected PTH1R indicate that activation of PLC, which can lead to activation of PKC via generation of inositol trisphosphate and diacylglycerols, requires that the N terminus of the ligand be intact (205). Also, PTH(1–31), found to lack PKC activation in some systems (122, 123), can nevertheless activate PKC, presumably via PLC, in others (206–208). Although available data are not fully congruent, one interpretation is that PTH1Rs can activate PKC(s) via at least two different mechanisms, one of which involves PLC and requires that the ligand have an intact N terminus, whereas the other, a PLC-independent mechanism, is triggered by more carboxyl ligand determinants, such as the region PTH(29–32) [or PTHrP(25–34) (209)]. These mechanisms may not both be active in all target cells.

Like other GPCRs, activated PTH1Rs appear to be phosphorylated by specific GPCR kinases, which then facilitate association with β-arrestin proteins (161, 163, 210–213, 215). β-Arrestins, in turn, may terminate receptor-G protein coupling and promote receptor endocytosis, although in the case of the PTH1R the specific role(s) of β-arrestin in receptor endocytosis and either degradation or recycling back to the surface remains unsettled and may differ in cells of different types (161–163, 212, 213, 215–217). β-Arrestins, when associated with certain GPCRs, also may support signaling functions, independent of classical G protein-mediated second-messenger generation, by serving as molecular scaffolds to assemble and activate kinases such as MAPKs and nonreceptor tyrosine kinases (218). It is not yet known whether such β-arrestin-mediated signaling can occur via PTH1Rs and contribute, for example, to the activation of MAPK observed in some PTH target cells (112–115, 117). Of particular interest are very recent observations that PTH1R internalization can be dissociated from receptor activation and that, in some cells, N-truncated PTH peptides such as PTH(7–34)

and PTH(7–84) may promote PTH1R endocytosis via a β-arrestin-independent, dynamin-dependent mechanism that is regulated (blocked) by interaction of the adapter protein NHERF1 with the cytoplasmic domain of the PTH1R (219). These novel findings raise the intriguing possibility that certain N-truncated PTH (CPTH) peptides, incapable of classical PTH1R second-messenger signaling but long enough to bind in some way to the PTH1R, could antagonize the actions of PTH1R agonists by inducing rapid receptor down-regulation, at least in cells lacking NHERF1. Although the role that this phenomenon plays in modulating PTH action *in vivo* remains to be determined, these observations suggest the possibility of a distinct cellular mechanism of action for at least some CPTH peptides, in addition to activation of CPTHRs.

C. Other members of the PTH/PTHrP receptor family

In 1995, Usdin *et al.* (220) reported that homology screening of a human brain cDNA library for other members of the class II GPCR family had led to isolation of a novel receptor, closely related to the PTH1R, which they named the "PTH2 receptor." Homologs of this receptor subsequently were identified in rat and zebrafish (221, 222). In rats, this receptor is expressed in discrete areas of the central nervous system, including hypothalamic, limbic, and sensory areas, especially in the spinal cord; parafollicular cells of the thyroid; peptide-secreting cells of the gastrointestinal tract; somatostatin-rich pancreatic islet cells; pancreatic exocrine cells; cardiac and vascular endothelium; vascular smooth muscle; lung; placenta; testis; and the vascular pole of the renal glomeruli but, unlike the PTH1R, not in renal tubules or bone (220, 223, 224). Pharmacologically, the PTH2 receptor (PTH2R) also differs strikingly from the PTH/PTHrP receptor, now referred to also as the type-1 PTH receptor (PTH1R), in that it is activated by PTH but not by PTHrP (90, 220, 221, 225–228). This PTH selectivity mapped to differences at position 5 of the ligand (Ile^5 in hPTH *vs.* His^5 in hPTHrP) and position 23 (Trp^{23} in hPTH *vs.* Phe^{23} in hPTHrP), which affected activation and binding, respectively (225, 226). Thus when the PTH-specific residues at these two positions were substituted into hPTHrP(1–36), activity at the PTH2R was reconstituted. Photoaffinity cross-linking analyses suggest that the overall orientation of the ligand relative to the receptor protein is similar for PTH binding to PTH1Rs and PTH2Rs, although specific residues within the N terminus of the ligand may play different roles in activating one receptor *vs.* the other (90, 229).

Like the PTH1R, the PTH2R exhibits dual signaling in response to PTH(1–34), with generation of both cAMP and cytoplasmic Ca^{2+} transients (227, 230). PTH(1–34) is a relatively potent agonist for the hPTH2R, at least when this receptor is expressed at high levels in cultured cells, but this is not true of the rat PTH2R (221, 230). This suggested that PTH may not be the endogenous ligand for the PTH2R. Subsequent demonstration of a potent PTH2R-selective activating factor in bovine hypothalamic extracts (231) was followed by the isolation and identification of a 39-residue peptide termed tuberoinfundibular peptide of 39 residues, or TIP39, that shows limited amino acid sequence homology to bPTH and activates PTH2Rs but not PTH1Rs (232). Later isolation of human and mouse TIP39 genomic DNA and tissue expression analysis in the mouse confirmed that this is a secreted peptide that is highly expressed in testis and, at lower levels, in various central nervous system nuclei, liver, and kidney (233). On the basis of the localization of PTH2Rs and TIP39 in the central nervous system and recent neurobehavioral studies, it appears likely that one of the important actions of TIP39 is to facilitate the response to painful stimuli (234). The selectivity of PTH2Rs *vs.* PTH1Rs for TIP39 is dictated by interaction of the first six amino acids of TIP39 with the J domain of the PTH2R, because chimeric receptors consisting of the PTH2R J domain and the PTH1R N-terminal extracellular domain, but not the reciprocal chimera, mediate binding and activation by TIP(1–39), whereas an analog, TIP(7–39), binds poorly to the PTH2R but well to the PTH1R (235). In fact, TIP(7–39) and TIP(9–39) are highly effective PTH1R antagonists (233, 235, 236). These surprising results indicate that the C-terminal portion of TIP39 can bind well to the PTH1R, presumably via interaction with its extracellular domain (237), but that this affinity is overridden by a conformational incompatibility between the J domain of the PTH1R and the N-terminal six residues of TIP39.

A third type of PTH receptor, termed the "type-3 zPTH receptor" or "zPTH3R", was cloned from a zebrafish cDNA library (222). At the level of amino acid sequence, this receptor is more closely related to mammalian PTH1Rs than PTH2Rs yet clearly is different from the zebrafish PTH1R, which was isolated concurrently (222). When expressed in mammalian (COS-7) cells, the zPTH3R activates adenylyl cyclase but not PLC and exhibits 20-fold higher affinity and potency with hPTHrP(1–36) than hPTH(1–34). Subsequent analysis, however, indicated that rat PTH activates the zPTH3R with higher potency than PTHrP, suggesting that this receptor probably is not preferentially responsive to PTHrP peptides (238). The importance of the PTH3R to human physiology is uncertain, however, because no evidence has been produced to date for the existence of a mammalian homolog of this receptor.

Evidence exists for other types of receptors that recognize N-terminal peptides of PTH or PTHrP but are less well characterized. Thus, Orloff *et al.* (239) reported sensitive cytosolic Ca^{2+} responses to both hPTHrP(1–36) and hPTH(1–34) (EC_{50} = 50–80 pM), without corresponding activation of adenylyl cyclase, in a series of human squamous cell carcinoma and keratinocyte cell lines. Specific binding of radioiodinated [Tyr^{36}]hPTHrP(1–36), competed similarly by hPTHrP(1–36), hPTHrP(1–74), hPTH(1–34), and bPTH(1–84) and of relatively low affinity (IC_{50} = 100–300 nM), was observed also, but only in some of the squamous cell lines. Efforts to identify receptor mRNA transcripts using PTH1R probes revealed atypical hybridizing bands but no clear evidence for PTH1R expression. Moreover, PTH1Rs transfected into squamous cells did elicit the expected cAMP response (240). Similar findings were obtained using a rat insulinoma cell line (241). Evidence of a brain receptor specific for PTHrP has been provided by Yamamoto *et al.* (242, 243), who observed that PTHrP(1–34), but not rat or human PTH(1–34), PTHrP(7–34), or hPTH(13–34), stimulated release of arginine vasopressin from slices of rat supraoptic nucleus. This effect was dose

dependent (0.1–1000 nM) and associated with a modest increase in cAMP [not seen with PTH(1–34)]. Both responses were blocked by PTHrP(7–37). PTHrP, but not PTH, showed specific binding to membranes that was of relatively low affinity (IC_{50} = 100 nM) and was competed by PTHrP(7–37) but not by PTH. None of these responses are readily explained by the known properties of the cloned PTH receptors isolated to date.

IV. PTH Secretion and Metabolism

A. Immunochemical heterogeneity of PTH in plasma

The first observation that different forms of PTH are present in blood was published in 1968 in a prescient paper by Berson and Yalow (244). These authors demonstrated that estimates of the concentration of PTH immunoreactivity present in human plasma and of the disappearance half-time of immunoreactive PTH from plasma after parathyroidectomy differed strikingly depending on the particular antiserum used in the immunoassay. They also observed that clearance of hormone was slower in patients with renal insufficiency and suggested that metabolic alteration of the hormone might account for the immunochemical heterogeneity (244). Shortly afterward, gel filtration experiments revealed that immunoreactive PTH present in human and bovine parathyroid gland extracts coeluted with native intact bPTH, as did hormone secreted *in vivo* from bovine parathyroids or human parathyroid adenomas, in contrast to PTH found in the peripheral circulation, much of which eluted from Biogel P-10 at a smaller molecular weight (245). This was the first direct evidence for the presence of PTH fragments in human and bovine blood, and the collective observations suggested that these fragments were produced mainly by cleavage of intact hormone after its release from the parathyroid glands. Closer analysis of venous effluent from patients with parathyroid adenomas, however, using fractionation by Biogel P-100 gel filtration and region-specific RIAs, provided evidence for the direct release of PTH fragments by the parathyroid glands (246). Moreover, the majority of secreted PTH fragments detected in the parathyroid venous blood samples were reactive in a RIA specific for the C-terminal region of PTH, whereas relatively little fragment immunoreactivity was detected in the same low-molecular weight fractions by a RIA specific for the N-terminal region (246, 247). Other laboratories quickly verified that hormonal fragments could be detected in human blood and, indeed, that multiple immunoreactive forms of PTH were detectable in plasma (248–250). It was noted also that immunoreactivity eluting from gel-filtration columns in the position of intact PTH disappeared rapidly after parathyroidectomy, whereas that eluting as smaller fragments persisted for longer periods (248). Silverman and Yalow (249) noted the presence of three hormone peaks on Sephadex G-100 filtration that were detected differentially by various antisera. They also observed that the fraction containing the smallest PTH fragments exhibited a much longer half-life in uremia, and that the antiserum detecting this material was most advantageous for the diagnosis of primary hyperparathyroidism. This latter observation ushered in a period of several years during which RIAs directed against different portions of the PTH molecule were tested for their respective clinical advantages, a detailed discussion of which is beyond the scope of this review. Direct iv infusion of exogenous intact bPTH into calves to constant plasma concentrations also revealed the accumulation of relatively long-lived C-terminal fragments that were generated by peripheral metabolism of the administered intact hormone (251). Thus, early evidence revealed that hormonal fragments in plasma could arise either by direct secretion from the parathyroid glands, or by peripheral metabolism of the hormone.

A different question was raised by the detection, in 1973, of N-terminal fragments in the circulation that were biologically active, as determined by monitoring renal adenylate cyclase activity (252). This and other immunochemical evidence for such circulating, short-lived N-terminal fragments (249, 250, 253, 254) led to the hypothesis that secreted PTH might have to be cleaved into fragments in the periphery before its ultimate actions in target tissues. A similar conclusion had been reached by Parsons and Robinson (255) in their studies of perifused feline bone. This theory subsequently was disproven, however. Thus, Goltzman *et al.* (256) demonstrated that intact bPTH could activate adenylate cyclase in renal cortical membranes and fetal rabbit calvarial bone without prior cleavage to fragments (73). The same was later shown with respect to release of cAMP and osteocalcin by intact bPTH from isolated perfused rat hindquarters (257). Intact biologically active radioiodinated bPTH also was shown to bind to canine renal membranes (140) and rat osteosarcoma cells (258) without prior cleavage to fragments. Thus, although synthetic N-terminal fragments such as hPTH(1–34) are highly active at PTH1Rs, conversion of PTH to such smaller fragments is not required for activity, nor is there any direct evidence that such fragments are produced *in vivo* by metabolic cleavage or gland secretion (see *Section IV.E.1*). Thus, such N-terminal fragments are not physiological agonists of the PTH1R.

B. Sources of circulating C-terminal PTH fragments

1. Secretion by the parathyroid glands. It has been firmly established that the parathyroids secrete C-terminal fragments of PTH as well as the intact hormone. The initial evidence for this came from use of assays specific for different regions of the hormone molecule to demonstrate C-terminal PTH fragments in venous effluent of parathyroid tumors (246, 259). Subsequently, Mayer *et al.* (260) determined by direct sampling of parathyroid venous blood of calves that, similar to the finding in patients with hyperparathyroidism, venous effluent from normal calves contained not only PTH, but also a major amount of C-terminal fragments. Indeed, these CPTH fragments were secreted in greater amounts than intact hormone, and the relative amounts of C-fragments increased with induced hypercalcemia (260). These results were consistent with earlier *in vitro* studies showing that most initially synthesized PTH is degraded intracellularly (261, 262), although neither PTH mRNA translation nor conversion of prePTH to proPTH is regulated by calcium (261, 263–265). Studies of cultured or perifused bovine parathy-

roid tissue *in vitro* also showed that CPTH fragments are present in parathyroid cells and are secreted directly from the glands (266–272). This was further supported by extensive chemical analysis of specific CPTH fragments produced by organ-cultured parathyroid tissue (273, 274). The ability of high extracellular calcium to augment release of CPTH fragments relative to that of intact PTH also has been repeatedly confirmed (272, 273, 275–278).

2. Hepatic proteolysis of intact PTH. Early work by Fang and Tashjian (279) showed that the liver contributes substantially to the clearance of circulating intact PTH, and this was confirmed by numerous subsequent analyses in several species, including humans (280–285). The liver can extract biologically active PTH(1–34) as well (286–288), when this peptide is administered exogenously. PTH has been shown to activate adenylate cyclase in liver (286, 289, 290), but direct hepatic actions of PTH are not known to be involved in systemic calcium homeostasis. Such hepatic actions of PTH, presumably mediated by the PTH1R, could reflect physiological autocrine actions of PTHrP. A more likely hepatic role in calcium and bone homeostasis, for which there now is considerable supporting evidence, relates to the uptake of intact PTH and its proteolysis by Kupffer cells to generate various circulating CPTH fragments (284, 291–293). Hepatic production of CPTH fragments (282) requires initial uptake of intact hormone by a mechanism that recognizes determinants present within PTH(28–48) but not PTH(1–34) (294). This fits with autoradiographic studies showing that intact PTH(1–84) binds to Kupffer cells, whereas PTH(1–34) does not (295). On the other hand, both PTH(1–84) and PTH(1–34) do bind to hepatocytes and sinusoidal cells (295). Some of the CPTH fragments generated by Kupffer cells are released back into the bloodstream, where they are not subject to further hepatic clearance (294) but rather are removed mainly by the kidneys (282) (see *Section IV.C*). Studies in hepatectomized *vs.* nephrectomized rats showed that the liver is the principal source of circulating CPTH fragments that result from peripheral metabolism of intact PTH (296).

Canterbury *et al.* (297) were the first to study liver metabolism of intact PTH in perfused rat liver, and they detected generation of N-terminal PTH fragments by using RIA after Biogel P-10 gel filtration. A fragment peak with a molecular weight of approximately 3500 Da was detected that was relatively enriched in N-terminal PTH immunoreactivity and could activate adenylate cyclase, similar to eluted peaks of intact bPTH or bPTH(1–34) (297). Subsequently, however, Daugaard *et al.* (298, 299) found in similar experiments that only C-terminal, biologically inactive fragments were generated during liver perfusion, as analyzed using HPLC fractionation. Daugaard suggested that differences in the purity of hormone preparations and the methods for analysis of fragments may have been responsible for the discrepant results. As discussed further below (*Section IV.E.1*), available evidence indicates that the N-terminal portion of the hormone is degraded locally by Kupffer cells and that N-PTH fragments do not reemerge from the liver into the circulation (292, 300, 301).

C. Renal clearance of PTH and PTH fragments

PTH immunoreactivity disappears more slowly from blood in humans with renal insufficiency (244, 302–304) and in nephrectomized animals (282, 284, 305, 306), and direct analysis of the fate of radiolabeled or immunoreactive PTH has confirmed the crucial role of the kidneys in clearance from blood of both intact hormone and, especially, CPTH fragments (248, 249, 282, 302, 306–310). A portion of intact PTH is cleared from blood by renal mechanisms that do not involve glomerular filtration, termed "peritubular uptake" by Martin *et al.* (306). This route is selective for PTH or PTH fragments capable of binding to the PTH1R (306, 311) and may involve receptor-triggered endocytosis at the basolateral surfaces of renal epithelial cells (145). The bulk of the hormone, however, is cleared by glomerular filtration and then is actively reabsorbed by the tubules (298, 306, 308). Recent studies implicate the megalin/cubilin endocytic system in the apical renal tubular epithelial clearance of PTH from tubular urine (312); this system is an important renal tubular scavenger receptor mechanism responsible for limiting renal clearance of low-molecular weight proteins. This PTH1R-independent mechanism is consistent with observations that overall clearance of biologically active PTH preparations does not differ from that of biologically inactive preparations (313, 314).

Although the involvement of the kidneys in clearance of PTH from the circulation is unequivocal and explains the disproportionate elevation of CPTH fragments observed in renal failure, it is unlikely that the kidneys are an important source of circulating CPTH fragments. Analysis of PTH metabolism by isolated perfused kidneys has produced conflicting results on this point (298, 299, 309, 315), but studies of acutely hepatectomized *vs.* nephrectomized rats demonstrated that the liver, not the kidneys, is the principal source of CPTH fragments in blood (296, 300).

Thus, both the liver and kidneys participate in clearance of circulating intact PTH, but only the liver generates, via the action of endopeptidases expressed by Kupffer cells, CPTH fragments that can then reenter the circulation (without accompanying N-PTH fragments). These CPTH fragments, like those secreted from the parathyroid glands, then undergo predominantly renal clearance.

D. Regulation of circulating C-terminal PTH fragment concentrations by serum calcium

By 1979 it was known from experiments in animals and humans that the relative contributions of intact PTH and hormonal fragments to PTH immunoreactivity in blood are regulated by calcium and influenced by renal failure (260, 304). Mayer *et al.* (260) provided the most definitive early data regarding the effect of blood calcium on secretion of PTH fragments by studying catheterized calves, where parathyroid effluent could be directly sampled under highly controlled conditions. These workers showed that C-terminal fragment secretion was responsible for the major portion of PTH immunoreactivity in hypercalcemia, whereas intact hormone was by far the major species of glandular output in hypocalcemia. As already noted above, this was subse-

quently confirmed by direct *in vitro* analysis of PTH peptide content and secretion from cultured or perifused parathyroid tissue (272, 273, 275, 276, 278).

At the same time, immunochemical analysis of circulating PTH in humans also documented that the ratio of CPTH fragments to intact hormone in peripheral blood is directly related to the calcium concentration (304, 316). Subsequent detailed analyses, using region-specific antibodies, have been carried out for the most part by the laboratory of D'Amour (317–323). In studies of normal human subjects in which parathyroid function was stimulated acutely by EDTA-induced hypocalcemia or suppressed by calcium infusion, these investigators, using RIAs specific for intact hormone *vs.* mid- or late-carboxyl PTH fragments, found that the regulation of intact hormone and hormonal fragments during hypo- and hypercalcemia differed (317–319). During acute hypocalcemia, intact PTH increased in serum 5- to 6-fold, and mid- and late-CPTH to a lesser extent, but these same CPTH fragments remained the predominant forms of PTH in blood. In response to acute hypercalcemia, intact PTH was suppressed 4- to 5-fold, whereas mid- and late-CPTH fragments declined only 30–50%, such that the latter became even more predominant relative to intact PTH (*i.e.*, 10-fold or higher in relative molar concentrations). Directionally similar calcium-dependent changes in ratios of mid- and late-CPTH fragments to intact hormone were observed in patients with primary hyperparathyroidism, but the apparent set point for calcium regulation was higher, such that higher serum calcium levels were required than in normal subjects to achieve the same ratios of CPTH/intact PTH (320). One notable aspect of this work was the finding that the ratio of mid-CPTH fragments to late-CPTH fragments was directly related to blood calcium concentration (318). This suggests that calcium may regulate the pattern of PTH proteolysis to produce relatively more CPTH fragments that are truncated at both their N and C termini. Experiments performed in dogs, designed to apply chronic stimulation or suppression of parathyroid function, have shown that CPTH fragments are more readily generated during hypercalcemic challenge after the glands have adapted to a chronic suppressive influence [such as 1,25-$(OH)_2D_3$ administration] (321, 322). Conversely, CPTH fragments are less easily produced, following the same acute hypocalcemic challenge, after a prolonged interval of parathyroid stimulation (as by calcium and vitamin D deficiency or after partial parathyroidectomy), consistent with time-dependent adaptation of intraparathyroidal peptidase activity (322, 323). In all of these investigations, a component of nonsuppressible intact PTH was noted during hypercalcemia, although it now is clear that immunoassays previously thought to be specific for the intact hormone may also detect long CPTH fragments [such as PTH(7–84)] (see *Section IV.E*). Thus, calcium-dependent excursions in ratios of secreted CPTH fragments:intact PTH may be even greater than suggested by these studies.

It seems clear that the secretion of CPTH fragments relative to intact PTH is regulated positively by extracellular calcium and that this likely contributes to the altered patterns of immunoreactive PTH peptides present in peripheral blood at different levels of blood calcium. However, the possibility that peripheral metabolism of PTH also may be regulated by changes in blood calcium remains unsettled. Work in rats and dogs has shown that the overall clearance rate of exogenously administered PTH is not affected by blood calcium (301, 314, 324, 325). Similarly, Oldham *et al.* (283) found no relation between serum calcium and the transhepatic arteriovenous gradient of PTH immunoreactivity in a small group of patients with primary hyperparathyroidism, although there was evidence of a calcium-dependent increase in renal extraction. Daugaard *et al.* (326), on the other hand, reported that hepatic extraction of intact PTH was accelerated 60% by increasing calcium concentration in the perfused rat liver system, but there was no evidence of a change in the efficiency of proteolysis to CPTH fragments. Earlier experiments performed by Canterbury *et al.* (297), in contrast, had indicated that the rate of cleavage of PTH by perfused rat livers was accelerated at low perfusate calcium concentrations. In intact anesthetized dogs, D'Amour *et al.* (327) found evidence of hepatic extraction of CPTH fragments that was suppressed by induced hypercalcemia. These authors concluded, however, that the elevated ratio of CPTH to intact PTH seen during hypercalcemia was due mainly to corresponding differences in rates of secretion rather than differences in metabolic clearance. With respect to renal clearance and proteolysis of PTH, Daugaard *et al.* (326) observed no relation between perfusate calcium and extraction of PTH by isolated perfused rat kidneys, nor were any CPTH fragments delivered into the perfusate. Hruska *et al.* (309), in contrast, found increased fragment production at low calcium in perfused canine kidneys. As noted by Daugaard, however, the latter observations may have been due to decreased glomerular filtration rates at higher calcium levels. Regulation of renal PTH proteolysis is of uncertain significance, however, because renal metabolism of PTH does not contribute significantly to the circulating pool of CPTH fragments (296, 300), which are derived principally from hepatic cleavage and parathyroid secretion.

In summary, whereas total immunoreactive PTH concentrations decline during hypercalcemia, the ratio of CPTH fragments to PTH in blood is positively correlated with serum calcium. This is associated with a calcium-dependent increase in secretion by the parathyroid glands of CPTH fragments relative to intact PTH. Although the overall metabolic clearance rate of PTH is not altered by changes in blood calcium, the possibility that calcium may regulate the rate or nature of hormonal proteolysis in the liver, and thereby contribute to an altered pattern of circulating PTH fragments, has not been excluded.

E. Nature of PTH fragments in blood

1. Circulating forms of N-terminal PTH. The almost exclusive form of circulating PTH capable of activating PTH1Rs and thereby exerting the classical actions on calcium homeostasis in kidney and bone is the intact hormone, as shown by experiments in humans, rat, and bovine species. Some studies, involving the use of region-specific immunoassays, chromatography of serum, or both, have pointed to the presence of low levels of circulating N-terminal PTH fragments, mainly in subjects with hyperparathyroidism, renal insufficiency, or both (249, 250, 252–254, 304, 305, 328, 329). Small

amounts of immunoreactive or bioactive PTH N-fragments have been reported also in parathyroid venous effluent or perfusate (246, 259, 260, 268, 328), again mainly with adenomatous or hyperplastic parathyroid tissue. Others, however, using direct chemical or radiochemical methods, have observed no secretion of N-terminal PTH fragments from parathyroid tissue (273, 274). All of these analyses have been plagued, to a greater or lesser extent, by issues of assay sensitivity and specificity, and in most cases the possibility of postcollection proteolysis *ex vivo* was not rigorously excluded. Thus, small PTH fragments reported in one study were observed also in hypoparathyroid serum in which intact PTH was entirely absent (304). In another well-controlled study involving use of a renal cytochemical bioassay and an immunoassay with predominant (but not exclusive) N-terminal specificity, low-molecular weight bioactivity was detected in uremic (but not normal) plasma and in parathyroid venous effluent, but there was no coeluting PTH immunoreactivity (328). The latter study did indicate that in normal subjects, at least 85% of plasma bioactivity coeluted with intact PTH.

The possibility that circulating bioactive N-terminal PTH fragments might result from postsecretory cleavage in peripheral tissues was raised by studies showing production of such fragments by perfused liver (297) or kidney (309), although others have not successfully replicated these results (299, 326, 330). Using bPTH(1–84) labeled to high specific activity at N-terminal methionines (positions 8 and 18) and HPLC resolution of radioactive peptides, Bringhurst *et al.* (292) showed that N-terminal fragments are produced during the endopeptidic cleavage of PTH by isolated rat Kupffer cells, but, unlike the corresponding CPTH fragments, are rapidly degraded. Moreover, subsequent studies *in vivo* using the same tracer, administered by continuous iv infusion to steady-state plasma concentrations, showed no accumulation of ^{35}S-labeled N-terminal PTH fragments in blood of normal, nephrectomized, hepatectomized, nephrectomized/hepatectomized, thyroparathyroidectomized/hypocalcemic or vitamin D-intoxicated/hypercalcemic rats, or in rats chronically maintained on either low- or high-calcium diets, under circumstances where the limit of detection of such fragments was 0.1 pM (300). These authors concluded that peripheral metabolism of PTH does not result in formation of measurable quantities of circulating PTH N-fragments under physiological or pathological circumstances. Thus, although yet to be proven definitively, it seems likely that, under physiological conditions, the holohormone, PTH(1–84), is the only circulating form of PTH with an intact N terminus and PTH1R bioactivity, as assessed by adenylate cyclase or cytochemical bioassay, or by known structural PTH sequences that interact with the N-terminal receptor. In particular, there is no direct evidence that N-fragments such as the PTH(1–34) peptide, widely used as a laboratory agonist for the PTH1R and as a pharmaceutical therapy for osteoporosis, exist naturally *in vivo*. In renal failure, especially with concomitant hyperparathyroidism, it is possible that low levels of N-terminal PTH fragments are produced and persist in the circulation in association with delayed renal clearance of hormone.

2. Circulating C-terminal fragments of PTH. As already reviewed, numerous analyses of human, rat, bovine, and porcine plasma have indicated that relatively high concentrations of circulating heterogenous C-terminal fragments exist under steady-state conditions. These fragments do not interact with the PTH1R and are therefore inactive in classical terms. It is likely that many of these fragments do possess biological activity, however, as discussed below.

Most available data concerning the nature of circulating CPTH fragments have been obtained using region-specific immunoassays (with or without preliminary gel-filtration chromatography) for which the reactive epitope(s) within the PTH molecule have been only crudely characterized. Efforts to more precisely define the structures of CPTH fragments in blood were initiated by Segre *et al.* (331, 332), who administered bPTH(1–84), radioiodinated at Tyr^{43}, to dogs and rats, isolated the resulting large radiolabeled CPTH fragments by gel filtration of plasma, and subjected these fractions to automated sequential Edman degradation to define the N termini of these CPTH peptides. These experiments demonstrated the presence in blood and liver (284) of multiple CPTH fragments, initially corresponding to those with N termini at positions 34 and 37 and followed by the appearance of additional fragments with N termini at positions 38, 40, and 43 of the bPTH sequence. Using organ ablation, this group also showed that these fragments arose exclusively from the liver and not from the kidneys, the other major site of clearance of PTH (296, 300). Although the ^{125}I-bPTH used by Segre *et al.* was biologically inactive (due to oxidation of N-terminal methionines during the iodination reaction), subsequent studies, using biologically active [^{3}H-Tyr^{43}]bPTH(1–84), documented major large circulating CPTH fragments with N termini at positions 34, 37, 41, and 43, with small amounts of additional fragments ending at positions 35 and 38 (300). Analogous fragments were found in rat liver extracts and could be produced by incubation of ^{125}I-bPTH or [^{3}H-Tyr^{43}]bPTH with isolated hepatic Kupffer cells *in vitro* (284, 291, 292). It was clear from the studies with Kupffer cells that these N termini corresponded in most cases to more than one peptide structure, because, for example, peptides with the same N termini (at positions 34, 35, 37, 38, and 41) could be found in different, widely separated HPLC fractions, the most likely explanation for which would be CPTH fragments with the same N termini but with different C termini (300). Other workers, using biologically active ^{125}I-bPTH and Kupffer cells prepared by different methods, identified the major CPTH fragments generated as having N termini at positions 35 and 38 (293), fragments which they also showed could be generated by incubation of the labeled PTH with purified cathepsin D (333). Clearance of CPTH fragments occurs mainly via glomerular filtration, as first inferred from very early studies (244) and later shown using purified ^{125}I-CPTH fragments (310). The clearance of C-terminal fragments from plasma has been studied in the rat (310). In normal rats radioiodinated C-terminal fragments were extracted by kidneys (33%), muscle (16%), bone (7%), liver (<3%), and other tissues (<1%). In nephrectomized rats, 25% of C-terminal fragments were cleared in muscle, 10% in bone, and 7% in liver, with less than 1% in other tissues. Thus,

nonrenal tissues can increase their ability to remove C-terminal PTH fragments in renal failure (310).

Analogous studies of the chemical nature of CPTH peptides secreted by porcine, bovine, or human parathyroid tissue have identified, remarkably, very similar N termini to those produced by hepatic proteolysis of intact PTH. Thus, Morrissey *et al.* (273), using a combination of microsequencing of radiolabeled peptides and tryptic peptide analysis, identified porcine (p)PTH(34–84) and pPTH(37–84) as secretory products of cultured porcine parathyroid cells, with pPTH(37–84) as the major moiety (2:1 molar ratio). Using N-terminal radiosequencing, MacGregor *et al.* (274) subsequently reported production by cultured bovine parathyroid cells of CPTH fragments having N termini at positions 24, 28, 34, 37, and 43 of bPTH, of which the putative cleavage at 23–24 was the earliest observed (within minutes) and the least suppressed by high medium calcium concentrations. The same group also reported secretion by human parathyroid cells of CPTH fragments with N termini at positions 24, 28, and 34 (334).

Thus, work performed more than 10 yr ago had identified the N termini of the principal CPTH fragments secreted by the parathyroid glands and shown that they were very similar, if not identical, to those generated by hepatic metabolism of circulating intact hormone and released back into the blood. It is important to recognize, however, that the precise structures of all circulating CPTH fragments have not yet been fully defined in any species. Differences in immunoreactivity registered in assays with predominantly mid- or late-carboxyl reactivity strongly suggest the presence of multiple CPTH fragments, some of which may extend to, or close to, the C terminus of intact PTH, whereas others may have undergone substantial C-terminal cleavage to produce bitruncated "mid-carboxyl" fragments (270, 318). Although most of the CPTH fragments characterized to date are at least 50–70% as large as PTH(1–84), some small "late carboxyl-terminal" fragments also may exist in human plasma (335).

More recently, strong evidence emerged for the presence in human plasma of N-truncated CPTH fragments that are long enough to register in conventional two-site immunoassays for intact PTH but that lack the N-terminal serine necessary for both full bioactivity at PTH1Rs and reactivity in a novel two-site immunoassay that stringently requires an intact N terminus (336–338). These fragments likely were not detected in previous studies because of poor chromatographic resolution from intact PTH and the limited sensitivity of repetitive Edman degradation when the N terminus is far removed from the radiolabel being monitored. They accumulate disproportionately to intact PTH in renal failure where they may constitute up to 50% or more of total intact PTH immunoreactivity, *vs.* 15–20% in normal subjects (336). Neither the sizes nor precise structures of these fragments have yet been ascertained directly. They do elute from reverse-phase HPLC columns just before PTH, in the same position as hPTH(7–84), which has been used to model their possible biological properties (see *Section VII.D*). It is known that these extended CPTH fragments can arise both from peripheral metabolism of iv administered hPTH(1–84) in rats and via secretion from human parathyroid adenomas *in vitro* (339), properties they share with other CPTH fragments previously described. The ability to measure intact PTH separately from these long CPTH fragments that interfere in conventional two-site assays may be of increased clinical value in assessing parathyroid and bone status in patients with renal failure (340, 341).

V. Nonclassical Actions of PTH

A. Actions on the intestine

PTH has been shown to promote intestinal absorption of calcium (342). Since the discovery of regulated renal 1-hydroxylation of vitamin D and the hormonal nature of 1,25-$(OH)_2D_3$ (343, 344), it has been the prevailing view that the action of PTH on intestinal calcium absorption that can be observed *in vivo* is indirect, via regulation of the renal vitamin D 1-hydroxylase, and that parathyroid stimulation of 1,25-$(OH)_2D_3$-mediated calcium absorption is the only mechanism whereby calcium gain from the environment can be promoted (345).

However, in addition to the classical actions of PTH on kidney and bone, PTH may also act, at least in pathological or pharmacological situations, to regulate calcium metabolism via direct stimulation of intestinal calcium absorption. Recent experiments have provided evidence in support of a more direct action of PTH on the intestine, as recently reviewed by Nemere and Larsson (346). Thus, whereas normal blood concentrations of 1,25-$(OH)_2$-vitamin D are a prerequisite for such action, PTH can increase calcium absorption more rapidly than would be expected from its ability to induce the renal 1α-hydroxylase. Rapid stimulation of calcium transport by PTH has been observed in cultured cells and in perfused loops of chicken duodenum (346). Although yet to be proven directly, it is likely that these responses reflect activation of classical PTH1Rs, expression of which has been documented in intestinal cells (346). These recently appreciated direct effects of PTH on calcium absorption have not been as well studied as those on bone and kidney, and their role under physiological conditions remains unclear. These effects may reflect paracrine actions of PTHrP in the intestine; alternatively, or in addition, they could represent a physiological pathway complementary to the indirect renally mediated action of PTH on the intestine that could operate systemically in the presence of sufficient vitamin D and a functional renal 1-hydroxylase.

B. Actions on osteoclasts

The prevailing view of the action of PTH in stimulating bone resorption is that PTH acts indirectly via PTH1Rs expressed on osteoblasts and stromal cells to enhance production of receptor activator of nuclear factor-κB ligand, macrophage colony-stimulating factor and possibly other cytokines (*i.e.*, IL-1, IL-6, and TNF-α) and to reduce production of the antiresorptive protein, osteoprotegerin (347–351). Then, according to this view, these actions promote differentiation of osteoclast precursors and stimulate the resorbing activity of mature differentiated osteoclasts (348). This mechanism, involving an indirect action of PTH on osteoclastic bone resorption, does not require the expression of PTH

receptors on osteoclasts. As early as 1983, however, evidence was presented for the binding of intact PTH to osteoclasts. Thus, Rao *et al.* (352), using immunoperoxidase staining, observed binding of unlabeled bPTH(1–84) to multinucleated osteoclasts in rat bone. Binding of labeled bPTH(1–84) to isolated avian osteoclasts was reported by Teti *et al.* (353) and Agarwala and Gay (354); the former used an iodinated radioligand, and the latter a biotinylated probe. Furthermore, biological effects of PTH have been demonstrated in osteoclasts. Thus, May and Gay (355) observed stimulation of acid production by highly enriched osteoclast preparations at concentrations as low as 10^{-11} M bPTH(1–84). The foregoing studies were carried out with intact PTH probes, but one study demonstrated binding of radioiodinated hPTH(1–34) to isolated avian and rat osteoclasts (356). Another report demonstrated high-affinity binding of a ^{125}I-PTH(1–34) analog to granulocyte-macrophage colony-stimulating factor-dependent hematopoietic blast cells capable of differentiating into multinucleated osteoclasts (357). Furthermore, recent studies have detected PTH1R mRNA expression in mature osteoclasts, although here the translated protein may reside predominantly in a perinuclear location (358, 359). Finally, it has very recently been demonstrated by confocal microscopy that almost all tartrate-resistant acid phosphatase-positive osteoclasts in rat metaphyseal immunostain for PTH1R (360).

The possible functional role of PTH1Rs detected on osteoclasts and their precursors remains uncertain, however, because few have reported direct biological actions of PTH(1–34) on such cells (357, 361–363) and most available evidence indicates that the effects of PTH(1–34) on osteoclast differentiation and activity are indirect, as noted above for PTH(1–84) (347, 348). On the other hand, May and Gay (355) suggested that the early observations of PTH(1–84) binding to osteoclasts, especially those of Teti *et al.* (353), in which the observed binding affinity was much lower than expected for a PTH1R interaction, could have represented binding of PTH to osteoclast PTH receptors specific for the C-terminal portion of the hormone. This is of particular interest now, given the recent finding that CPTH fragments can interact directly with hematopoietic precursors to reduce their differentiation to osteoclasts (see Section *VII.D*).

C. Unique nonclassical actions of intact PTH

With respect to the classical PTH1R-mediated actions of PTH on calcium and bone metabolism, there is general agreement that the activity of PTH(1–84) is equivalent to that of synthetic N-terminal fragments such as PTH(1–34) (7, 69–73). This equivalence appears to pertain also to the anabolic effects of intermittently administered PTH and PTH(1–34) on bone (364–366). Indeed, the latter has recently been adopted for use in therapy of osteoporosis (367); hPTH(1–84) is also presently in clinical trials (368). On the other hand, certain nonclassical actions of PTH have been described in which the effects of the intact hormone seem to be distinctly different from those of PTH(1–34). Among the first of these was a report by Hruska *et al.* (369) that iv bPTH(1–84) (2.5 mg/kg) stimulated hepatic gluconeogenesis and alanine uptake in dogs within minutes, whereas bPTH(1–34) administered at the same dose exerted no effect. Later, in thyroparathyroidectomized dogs, Puschett *et al.* (370) found that PTH(1–34), but not a 3-fold molar excess of PTH(1–84), exerted an acute natriuretic and calciuric effect. In both of these studies, peptide bioactivity had been confirmed—*i.e.*, each peptide was shown to stimulate cAMP production in isolated canine renal tubules and cause phosphaturia *in vivo*. Martin *et al.* (371) reported that bPTH(1–84), unlike bPTH(1–34), could not activate adenylyl cyclase in the perfused canine hindlimb. This was somewhat reminiscent of earlier experiments performed by Parsons and Robinson (255), who found that bPTH(1–84) could not elicit rapid calcium release from an isolated perfused cat tibia if it was injected directly into the blood supplying the bone but could do so if administered to the animal whose circulation was being diverted to perfuse the bone. These latter experiments have not been explained but seem consistent with a need for preliminary processing or cleavage of the intact hormone or the possibility of differential access to cellular receptors via the capillary epithelium.

In an extensive series of studies directed at the contribution of PTH to the uremic syndrome, Massry and colleagues described various actions of PTH(1–84) that were not shared by PTH(1–34) (372–377). For example, Meytes *et al.* (372) observed that bPTH(1–84), but not bPTH(1–34), inhibited erythroid burst colony formation in cultured murine bone marrow, although both preparations were biologically active, as measured by cAMP stimulation in renal tissue. Similarly, PTH(1–84) but not PTH(1–34) was found to enhance neutrophil random migration (373) and elastase release (374) to elicit cytoplasmic Ca^{2+} transients in rat thymocytes (375) and adipocytes (376) and to stimulate the volume of pancreatic secretions (377). In other systems, PTH(1–34) was found to be active but significantly less potent than PTH(1–84). These included induction of chronotropic effects in rat cardiomyocytes (378); stimulation of cytoplasmic Ca^{2+} responses in rat pancreatic islets (379), cardiomyocytes (380), hepatocytes (381), and proximal tubular cells (382); and inhibition of human B cell proliferation (383). In many of these studies of Massry *et al.*, however, the action of PTH(1–84) could be inhibited by standard PTH1R antagonists, such as analogs of PTH(7–34), or by oxidation of the PTH(1–84), which destroys the two N-terminal methionines required for PTH1R bioactivity. Thus, the selective effects of PTH(1–84) observed in these studies seem most consistent with its action via PTH1Rs, although the absence of a comparable response to PTH(1–34) in these systems remains unexplained. It is noteworthy, however, that PTH(19–84), which cannot bind effectively to PTH1Rs, nevertheless did mimic the action of PTH(1–84) on neutrophil elastase, an assay in which PTH(1–34) was inactive (374).

Sun *et al.* (384), in 1997, reported that hPTH(1–84), continuously infused into rats for 3 d at a dose (7 pmol/h) that did not cause hypercalcemia, elicited a much greater increase in circulating fibronectin (6.4-fold *vs.* 2.2-fold) than did the same molar dose of infused hPTH(1–34). This was true although the two peptides reached the same steady-state molar concentration in plasma. A similar difference in fibronectin levels was seen in medium from ROS 17/2.8 cells cultured with these peptides for 3 d at concentrations between 10 pM and 100 nM. The hPTH(1–84) also augmented 1,25-$(OH)_2D_3$

levels *in vivo* more effectively than hPTH(1–34) (81 ± 2 *vs.* 65 ± 1 pg/ml), although the two peptides were equipotent in stimulating cAMP production by ROS 17/2.8 cells (384).

In summary, these various observations, involving diverse experimental systems and responses, have provided some evidence for differences in biological activity between intact PTH and synthetic N-terminal fragments such as PTH(1–34) that otherwise are believed to be fully equivalent agonists at the PTH1R. Whether these differences reflect involvement of multiple PTH receptor species, atypical preferential activation of PTH1Rs (or other PTH1R family members) by one or the other peptide in certain cell types, differences in *in vivo* or *in vitro* bioavailability or pharmacokinetics or technical issues has not been established. Nevertheless, these collective findings suggest that intact PTH and PTH(1–34) may not be bioequivalent in all cells or tissues.

VI. Receptors that Bind Specifically to the C-Terminal Region of PTH

A. Early studies of PTH binding to target tissues

During the late 1970s, there was increasing interest in studying PTH receptors by measuring hormone binding to target tissue membranes. In view of the emerging belief that PTH bioactivity resided in the N terminus of the hormone molecule, initial studies of PTH receptors used radiolabeled ligands based on PTH(1–34). Because conventional radioiodination methods resulted in loss of biological activity, radioligands were needed that retained full biological activity of the hormone with high specific radioactivity. Zull and Repke (385) used an acetamidation reaction to produce a biologically active tritiated derivative of bPTH(1–84). Radioiodinated ligands were preferred, however, because of their significantly higher specific radioactivity. Gentler radioiodination methods were either electrolytic (386, 387) or used the peroxidase reaction (388). With any of these radioiodination methods, the labeled hormones had to undergo final purification, either by adsorption to chicken renal membranes or by HPLC, for full receptor binding activity. The method of Rosenberg *et al.* (387) had the advantage of specifically targeting a 1:1 molar ratio of radioiodine to hormone. Also, with that method radioiodinated hormone was shown to retain full hormonal bioactivity in the conventional rat renal adenylate cyclase assay, the rat calvarial calcium release assay, and the chicken hypercalcemia bioassay (387).

Initial studies of renal receptors detected saturable high-affinity binding of N-terminal PTH ligands with dissociation constants in the low nanomolar range, tightly linked to adenylate cyclase [reviewed by Nissenson (389)]. Similar results were found in chicken renal plasma membranes (75), canine renal cortical membranes (74, 390), and rat renal membranes (391, 392). Analogous results were obtained with chicken calvarial osteoblasts (390, 393) and rat osteosarcoma cells (394).

B. Evidence for distinct binding sites for C-terminal PTH

1. Initial detection of PTH C-terminal binding using intact hormone radioligands. In 1980, using a cytochemical bioassay of guinea pig renal slices, Arber *et al.* (395) discovered that hPTH(53–84) elicited a unique late-occurring peak of glucose-6-phosphate dehydrogenase activity, whereas that produced by PTH(1–34) occurred much earlier. This observation, published in abstract only, went largely unnoticed. More interest in the activity of the carboxyl-terminal region of the hormone was aroused, however, when receptor binding experiments were carried out using intact hormone radioligands comprising the entire hPTH(1–84) sequence. A number of such experiments revealed that the competition for binding of intact hormone radioligand by unlabeled PTH(1–34) was incomplete. High concentrations of the biologically active N-terminal fragment could only partially displace binding of intact hormone, which implied that not all of the binding of intact hormone to target tissues involved the receptor for the N-terminal 34 residues of PTH and suggested the presence of functional binding determinants C-terminal to residue 34 of the hormone (140, 141, 258, 396).

2. PTH C-terminal binding sites in kidney. More direct evidence for the presence of distinct C-terminal PTH binding sites was first provided by McKee and Murray (142), who studied the binding of biologically active ^{125}I-bPTH(1–84) to chicken renal plasma membranes and directly tested the competition for intact hormone binding with a C-terminal fragment of PTH. Whereas binding studies of PTH(1–34) radioligands uniformly exhibited a single class of binding sites, kinetic analysis of intact hormone radioligand binding revealed a clearly biphasic Scatchard plot, indicating the presence of two distinct classes of binding sites. Competition curves with either unlabeled bPTH(1–34) or hPTH(53–84) clearly probed an N-terminal binding site on the one hand and a C-terminal site on the other. The N-terminal site was of high affinity (K_d = 1.21 nM), similar to previously published studies using specific N-terminal radioligands, and was tightly linked to adenylate cyclase activation. On the other hand, the C-terminal site was of lower affinity (K_d = 333 nM). Binding to the low-affinity C-terminal site differed in several other respects from that of the high-affinity N-terminal binding: it was specifically inhibited by 10 mM Mg^{2+}, it was reduced disproportionately after storage of membranes for 1 month at −70 C, and it took a significantly longer time for competition by unlabeled fragments to occur. Furthermore, it was not linked to adenylyl cyclase activation (142). Subsequent studies from the same laboratory using an intact cell system, the OK opossum kidney cell line, also indicated the presence of two distinct sites for the biologically active intact hormone radioligand, a high-affinity N-terminal binding site (K_d = 3.4 nM) linked to adenylyl cyclase activation and a lower-affinity C-terminal binding site not so linked [K_d = 623 nM (397)]. In both the broken cell chicken plasma membrane study and the intact OK cell system, ligand binding to the C-terminal sites could be specifically competed by hPTH(53–84) fragment. These C-terminal sites were numerous, because up to 50% of labeled intact hormone could be displaced from OK cells by unlabeled hPTH(53–84). In OK cells, the N-terminal fragment was a more potent activator of adenylyl cyclase than the intact 84-residue hormone by two orders of magnitude, consistent with competition between N- and C-terminal binding sites for intact hormone but not N-fragment; hPTH(53–84)

did not activate adenylate cyclase (397). The biological relevance of the renal binding sites for C-terminal PTH remains unknown, because additional analysis of possible renal actions of defined CPTH peptides has not been pursued directly since the early report of Arber *et al.* (395) in the renal cytochemical bioassay.

3. PTH C-terminal binding sites in bone. These studies in renal cells were quickly followed by experiments in bone cell lines using biologically active intact PTH radioligands. The Toronto group undertook analyses in the osteoblastic rat osteosarcoma cell line ROS 17/2.8. As in the kidney systems, kinetic analysis of the binding of intact biologically active bPTH to the ROS 17/2.8 cells revealed two classes of binding sites. However, there was one sharp difference between the kidney and skeletal data; in the ROS 17/2.8 cells, much more of the intact hormone binding to these cells of skeletal origin involved the C-terminal binding sites, as indicated by the ability of saturating doses of hPTH(53–84) to compete for as much as 72% of radioligand binding (Fig. 2) (398). Again, the N-terminal sites had higher affinity (K_d = 19 nM) than the C-terminal binding sites (K_d = 210 nM). Whereas N-terminal PTH binding was linked to adenylyl cyclase activation, C-terminal binding was not. Rao and Murray suggested that the C-terminal sites might be coupled to an unknown biological activity and suggested that additional studies with defined C-terminal fragments of PTH other than hPTH(53–84) be carried out (398). A later study of binding to ROS 17/2.8 cells of [^{35}S]hPTH(1–84), prepared by *in vitro* translation, largely confirmed the findings of Rao *et al.* Thus, Takasu *et al.* (399) observed an apparent K_d of approximately 200 nM for specific binding that was inhibited completely by hPTH(1–84), 70% by hPTH(53–84), but only 30% by hPTH(1–34). In these studies, in which the binding affinity of the intact hormone radioligand was somewhat lower, CPTH fragments hPTH(35–84) and hPTH(69–84), but not hPTH(70–84), hPTH(71–84), or hPTH(53–83), when present at 3300 nM, also significantly inhibited [^{35}S]hPTH(1–84) binding and showed additivity with displacement caused by high concentrations of hPTH(1–34).

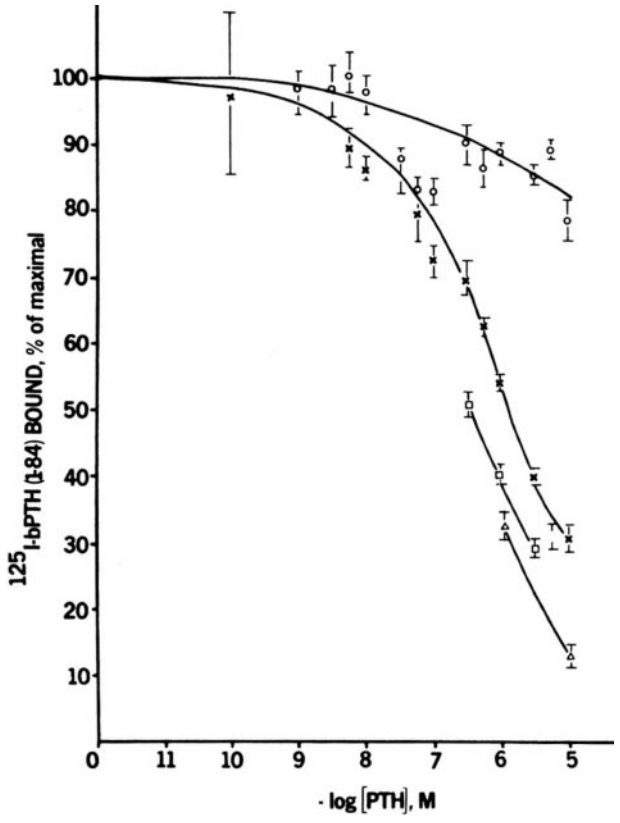

FIG. 2. Inhibition of ^{125}I-bPTH(1–84) binding by N-terminal and C-terminal PTH fragments. Competitive inhibition of [^{125}I]bPTH(1–84) binding to ROS 17/2.8 cells by unlabeled bPTH-(1–84) (△), hPTH-(53–84) (x), bPTH-(1–34) (□), and hPTH-(53–84) + bPTH-(1–34)(○). The labeled hormone was added to the confluent cultures of ROS 17/2.8 cells together with unlabeled hormones. Incubations were carried out at 22 C with constant shaking for 2 h. The washed cells were solubilized with NaOH-0.08% sodium dodecyl sulfate, and the radioactivity was counted in an automatic γ-counter. Each point represents the mean ± SEM of four determinations from at least two to three experiments. [Reprinted with permission from L. G. Rao and T. M. Murray: Endocrinology 117:1632–1638, 1985 (398). © The Endocrine Society.]

Demay *et al.* (141), in comparing binding of lactoperoxidase-labeled ^{125}I-bPTH(1–84), before and after oxidation of the N-terminal methionines required for PTH1R binding, also suggested that UMR 106 rat osteosarcoma cells expressed binding sites with specificity for the C terminus of PTH, although, curiously, hPTH(53–84) could not displace the ^{125}I-bPTH(1–84) radioligand, unlike the studies of Rao *et al.* (398) in ROS 17/2.8 cells. Later studies from the Toronto laboratory (400) found that with UMR-106 cells, hPTH(53–84) did compete for binding of ^{125}I-bPTH(1–84) but to a lesser degree than in their experiments with ROS 17/2.8 cells. The discrepancies between these reports may be explained by differences between these two osteosarcoma cell lines.

A significant advance was reported by Inomata *et al.* (401), who generated recombinant analogs of hPTH, [Tyr^{34}]-hPTH(19–84) and [Leu^{8,18}, Tyr^{34}]hPTH(1–84), which interact weakly or not at all with the PTH1R. When radioiodinated (at the Tyr^{34} residue substituted for Phe^{34}), each of these peptides exhibited specific binding to sites highly expressed on ROS 17/2.8 and on rat parathyroid-derived (PT-r3) cells that could be fully displaced by hPTH(1–84) or [Tyr^{34}]-hPTH(19–84) but not at all by PTH(1–34) (1 μM). Shorter CPTH fragments, such as hPTH(39–84) and hPTH(53–84), also displaced the CPTH radioligands, but with 10- to 50-fold lower affinity, whereas several other peptides, including hPTH(44–68), hPTHrP(37–74), and hPTHrP(109–141), showed no measurable binding. These data suggested that the CPTH receptor, unlike the PTH1R, was specific for PTH (*vs.* PTHrP) and that residues located in the 19–38 portion of the PTH ligand were important for binding. Interestingly, these binding sites, specific for the C-terminal portion of the hormone, were up-regulated on ROS 17/2.8 cells by exposure for 48 h to PTH(1–34), hPTH(1–84), or 8-bromo-cAMP, whereas treatment with either dexamethasone (which increases PTH1R expression in these cells) or hPTH(39–84) had no effect. Evidence of CPTH-specific binding, at lower levels, was found in UMR 106-01 cells and OK opossum kidney cells, but not in YCC squamous cell carcinoma cells, LLC-PK$_1$ renal epithelial cells, or SaOS-2 or MG63 human osteosarcoma cells. In a subsequent study, Divieti *et al.* (402) found no significant specific CPTH binding (<0.6%) in NIH-3T3,

HeLa, and BHK21 cells. The study of Inomata *et al.* (401) was particularly important also because it provided, for the first time, direct physical evidence of these CPTH binding sites. Thus, when the CPTH-specific radioligands were chemically cross-linked to plasma membranes of ROS 17/2.8 cells and analyzed by gel electrophoresis, two discrete bands were observed having molecular weights of approximately 90 and 40 kDa, respectively (including the radioligand mass of ~10 kDa). These were coordinately displaced by added nonradioactive PTH(1–84) at the same concentrations that were effective in the direct noncovalent equilibrium binding analyses. The significance of the smaller protein band observed in the ROS 17/2.8 cells is unclear, because only the larger band was observed in similar studies performed with the PT-r3 cells.

C. Demonstration of C-terminal PTH binding in the absence of PTH/PTHrP receptors

In 1996, the generation of mice with genetically ablated PTH1Rs (176) allowed, for the first time, a definitive approach to the question of whether binding of CPTH-specific radioligands and fragments to putative CPTH receptors (CPTHRs) requires the PTH1R in any way. Thus, starting with collagenase-digested bone cells from embryonic PTH1R-null mice, Divieti *et al.* (402) selected a series of conditionally immortalized clonal cell lines on the basis of their high expression of specific binding sites for the ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand. The resulting cell lines exhibited a uniform, stellate appearance and a pattern of gene expression consistent with an osteocytic phenotype. As anticipated, ligand binding analysis with ^{125}I-[Tyr^{34}]hPTH(19–84) confirmed the presence, in these osteocytic cells, of high numbers of C-terminal binding sites, in the range of 1–3 $\times$ 10^6 per cell. The CPTHR density on these cells was 4- to 5-fold higher than in ROS 17/2.8 cells or other (osteoblastic) clonal PTH1R-null cell lines that previously had been isolated from the same embryonic bone digests on the basis of high expression of alkaline phosphatase (403). Analysis of shorter CPTH fragments also confirmed the presence of important binding determinants within the 19–39 region of PTH, as previously reported (401). In addition, binding affinity of hPTH(28–84) was found to be similar to that of hPTH(39–84) and hPTH(53–84), *i.e.*, 20- to 30-fold lower than that of hPTH(1–84), hPTH(19–84), or hPTH(24–84), which showed IC_{50} values in the 10–30 nM range (404) (Fig. 3). This indicated that the basic sequence Leu^{24}-Arg^{25}-Lys^{26}-Lys^{27}, which is highly conserved among mammalian species (Fig. 1), contributes significantly to the binding affinity of PTH to the CPTHRs on these cells. Moreover, additional analyses showed that, whereas hPTH(53–84) binds to CPTHRs with reduced affinity (IC_{50} = 500 nM), the slightly shorter peptide hPTH(55–84) could not displace the radioligand at all, even at concentrations as high as 10 μM. Thus, the dibasic sequence Lys^{53}-Lys^{54}, also highly conserved (Fig. 1), constitutes a second important ligand domain necessary for optimal CPTHR binding. The same group also reported that a short fragment of PTH, PTH(24–54), comprised of these two "binding domains" and the intervening sequence, still could bind to CPTHRs on osteocytes and fully displace the iodinated PTH(19–84) tracer, although the affinity was quite low (IC_{50}, 10–20 μM) (404). Notably, the apparent affinity of this PTH(24–54) peptide was approximately 1000-fold lower than that of hPTH(24–84) (20 nM). This comparison highlights the fact that the region comprised by hPTH(55–84) must contain at least one critical determinant of ligand binding affinity to CPTHRs, although the hPTH(55–84) peptide itself displays no binding at all in the absence of more N-terminal contiguous sequence.

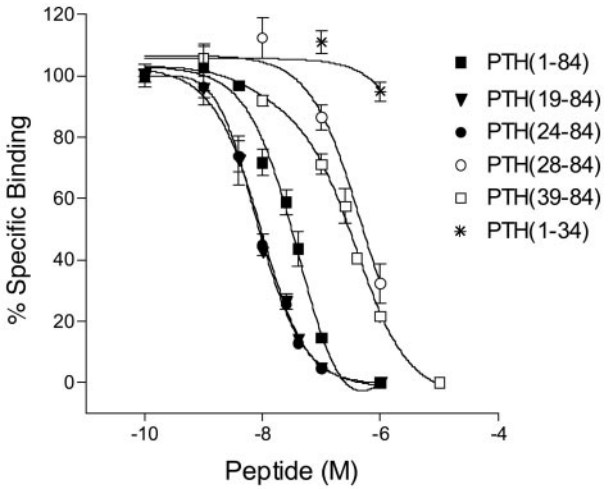

FIG. 3. Binding of N-terminally truncated hPTH fragments to CPTHRs on clonal PTH1R-null osteocytes. The hPTH peptides shown were tested for their ability to displace ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand from OC59 osteocytic cells. Results are shown as mean ± SD of the percentage of maximal specific binding observed in the absence of competing ligand (n = 3). [Reprinted with permission from P. Divieti *et al.*: Endocrinology 142:916–925, 2001 (402). © The Endocrine Society.]

These key features of ligand selectivity are shared in common by CPTHR sites expressed on several different clonal cell lines, including PTH1R-null osteoblasts (403) and chondrocytes (405) and PTH1R-expressing marrow stromal cells (406) (Fig. 4). Such observations suggest that CPTHRs expressed by skeletally derived cells are either identical or very similar, at least in terms of ligand selectivity. One disparity with previous work in PTH1R-expressing cells concerns the observation of Takasu *et al.* (399) that [^{35}S]hPTH(1–84) could be displaced from sites on ROS 17/2.8 cells by the peptide hPTH(69–84), whereas Divieti *et al.* (404) observed no displacement of ^{125}I-[Tyr^{34}]hPTH(19–84) from clonal bone cells, regardless of PTH1R expression, by peptides as short as hPTH(55–84) or hPTH(60–84). It remains possible that hPTH(69–84) will be found to displace ^{125}I-[Tyr^{34}]hPTH(19–84) from the PTH1R-null cell lines, because this peptide has not been directly tested [*i.e.*, perhaps the hPTH(60–68) domain is inhibitory to binding]. Alternatively, differences in cell systems or in features of the radioligands used (specific activity, presence of the Tyr^{34} residue or N-terminal extent) may underlie the apparent discrepancy. As noted below, the expression of CPTHRs on PTH1R-null bone-derived cell lines is associated with biological responses to PTH peptides that are observed at concentrations similar to those required for binding, consistent with the classical pharmacological definition of a receptor.

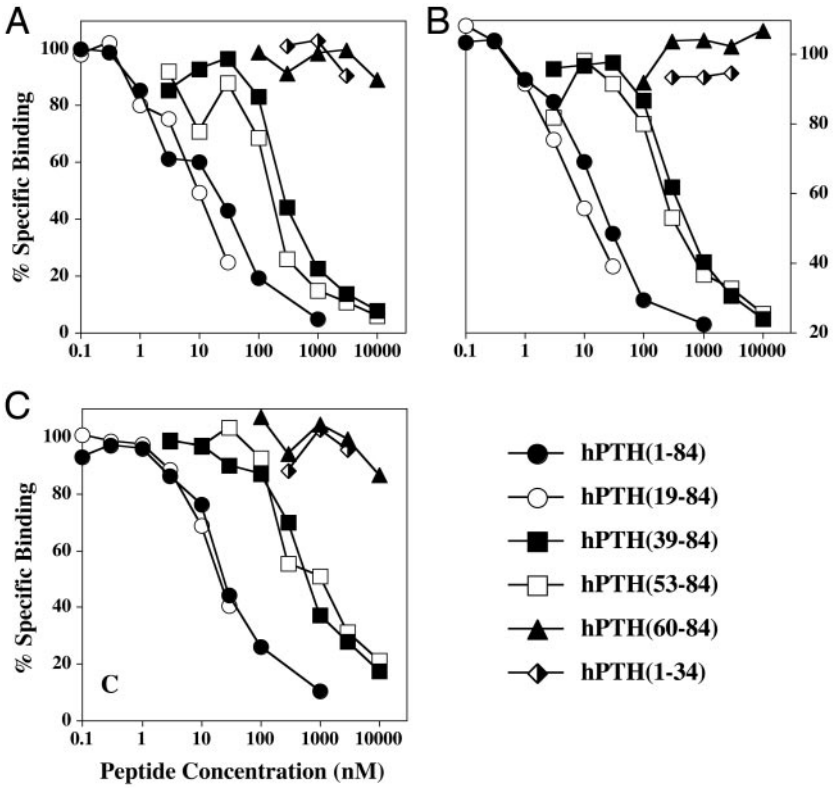

FIG. 4. Ligand selectivity of CPTHRs expressed by different cells of skeletal origin. Cells were incubated at 4 C overnight with ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand, with or without various synthetic hPTH peptides present at the indicated concentrations (nanomoles), as previously described (402). Binding is shown as a percentage of the specific binding observed with radioligand alone in clonal PTH1R-null HC-1 hypertrophic chondrocytes (A) (405), F1–14 PTH1R-null osteoblasts (B) (403), and MS1 marrow stromal cells (C) (406).

VII. Distinct Biological Activities of PTH C-Terminal Fragments

A. Initial evidence for biological activity of C-terminal PTH fragments in bone

During the late 1980s, the evidence from receptor binding experiments for distinct C-terminal PTH binding sites in both kidney and bone was not sufficiently compelling to engender wide acceptance of the concept of a biologically relevant CPTHR in PTH target tissues, especially because the C-region of the molecule was generally thought to be biologically inactive. It thus was of seminal importance that a biological activity in bone was discovered in 1989 by Murray *et al.* (407), who showed that the C-terminal fragment hPTH(53–84), the only synthetic CPTH fragment commercially available at the time, regulated alkaline phosphatase activity in rat ROS 17/2.8 osteosarcoma cells and did so in a manner opposite to that of bPTH(1–34). The enzyme activity was stimulated by doses of hPTH(53–84) as low as 0.01 nM (408), and the effect was only noted when the ROS cells were cultured in the presence of dexamethasone. Increasing doses of dexamethasone greater than 1 nM resulted in a dose-related increase in the stimulatory effect of hPTH(53–84) on enzyme activity, whereas a concomitant dose-related inhibition was seen with bPTH(1–34) treatment (Fig. 5) (408). Alkaline phosphatase stimulation in osteoblastic cells was the first specific biological activity attributed to a CPTH fragment. Additional work by the Toronto group demonstrated that hPTH(53–84) could regulate the expression of the alkaline phosphatase gene as well as that for osteocalcin, whereas hPTH(1–34) did not (409, 410). On the other hand, hPTH(1–34) stimulated expression of the gene for type I collagen, whereas hPTH(53–84) did not (410).

B. Structure vs. function of PTH C-terminal fragments on bone cells

These initial findings of Murray *et al.* (407) subsequently were confirmed and extended by Nakamoto *et al.* (411), who used the same cell system and observed that hPTH(53–84) (10^{-9} to 10^{-7} M), but not hPTH(39–68) or hPTH(71–84), augmented alkaline phosphatase activity. This suggested that the stimulatory effect of hPTH(53–84) could not be assigned to either its N- or C-terminal half, although neither of the two inactive peptides has been shown to bind effectively to CPTHRs. Interestingly, the hPTH(69–84) fragment was slightly inhibitory in these experiments, an effect that apparently requires the dipeptide moiety Glu^{69}-Ala^{70} [given that hPTH(71–84) was inactive]. Although the results of Murray *et al.* (407) with the corresponding bovine peptides showed that bPTH(1–34) and bPTH(1–84) inhibited alkaline phosphatase activity to the same extent, Nakamoto *et al.* (411) found that synthetic hPTH(1–84) inhibited alkaline phosphatase with slightly greater potency than did hPTH(1–34) and, furthermore, that the combination of the inhibitory hPTH(69–84) and hPTH(1–34) fragments mimicked the enhanced suppressive effect of the intact hormone. This additivity was not observed with hPTH(39–68) or hPTH(71–84). Moreover, the combination of hPTH(1–34) and hPTH(53–84) produced a response (modest inhibition) that was intermediate between the inhibitory and stimulatory effects, respectively, of either peptide alone (411). Many of these observa-

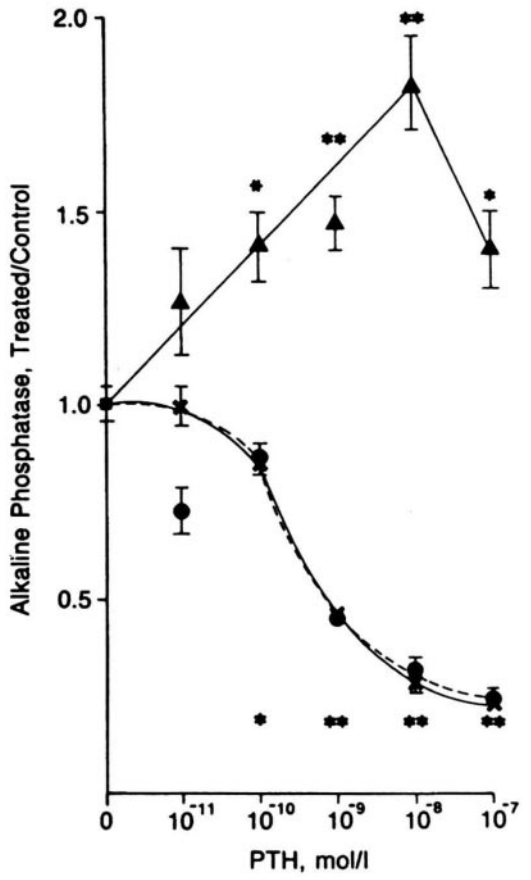

FIG. 5. Regulation of alkaline phosphatase activity in ROS 17/2.8 osteosarcoma cells. Stimulation and inhibition of alkaline phosphatase activity in dexamethasone-treated ROS 17/2.8 cells by increasing doses of PTH peptides. hPTH-(53–83) (▲) stimulated enzyme activity, whereas bPTH-(1–34) (●) and bPTH-(1–84) (x) inhibited it. Cells were plated in multiwell dishes and cultured for 3 d. The medium was then changed to fresh medium containing 2% fetal calf serum and varying doses of PTH peptides. Data were pooled from three experiments performed in triplicate. Significance of difference from control group: *, $P < 0.01$; **, $P < 0.001$. [Reprinted with permission from T. M. Murray *et al.*: Endocrinology 124:1097–1099, 1989 (407). © The Endocrine Society.]

tions, including the inhibitory effect of hPTH(69–84), on alkaline phosphatase activity were later reconfirmed in the ROS 17/2.8 cell system by Takasu *et al.* (399), who also reported new observations that hPTH(35–84) stimulated alkaline phosphatase, whereas both hPTH(70–84) and hPTH(53–83) were inactive. Collectively, these rather complex findings seem consistent with either the expression by the osteosarcoma cells of different classes of CPTHRs with discrete ligand selectivity, perhaps associated with changes in the clonal cell line after many additional passages, or the possibility that a single species of CPTHRs can be activated differently depending on the sequence and length of the particular CPTH peptide ligand applied or cell differentiation (see following paragraph).

Additional evidence of specific bioactivity of CPTH fragments in bone cells was reported by Fukayama *et al.* (412), who observed that hPTH(39–84), hPTH(44–68), and hPTH(53–84), each at 2.5×10^{-8} M, stimulated the uptake of extracellular $^{45}Ca^{2+}$ within 15 min of the addition to SaOS-2 human osteosarcoma cells. Nasu *et al.* (413) then reported, using UMR 106 rat osteosarcoma cells, that N-terminally truncated PTH fragments, such as PTH(35–84), PTH(53–84), and PTH(69–84) could increase the expression of type-1 procollagen mRNA. Notably, whereas PTH(1–34) inhibited procollagen-1 mRNA expression, the intact hormone had no effect, a finding at least consistent with the possibility that simultaneous activation of the two receptor sites (PTH1R and CPTHR) could lead to summation of the opposite biological responses to activation of each alone. In contrast to previous reports in ROS 17/2.8 cells (408, 410, 411), Nasu *et al.* (413) found that PTH(53–84) did not augment alkaline phosphatase activity in UMR 106 cells, although different experimental conditions were used (shorter exposure to peptides and no dexamethasone treatment). These investigators also reported an increase in mRNA encoding IGF binding protein-5 in UMR 106 cells in response to PTH(1–84), PTH(35–84), and PTH(53–84), although the shorter fragment PTH(69–84) was inactive. Stimulation of IGF binding protein-5 mRNA was seen also with hPTH(1–34), which indicated that this particular response is regulated similarly by CPTHRs and PTH1Rs in these cells (413). Differences between these findings and those of earlier reports may be explained by the likelihood that UMR-106 cells represent an earlier stage of osteoblastic differentiation than do ROS 17/2.8 cells. Furthermore, the studies of Nasu *et al.* (413) were conducted in the absence of dexamethasone, whereas Murray *et al.* (407, 408) observed the stimulatory effect of PTH(53–84) on alkaline phosphatase in ROS 17/2.8 only in the presence of this steroid. Corticosteroids are known to promote osteoblast differentiation and may strikingly alter osteoblast phenotype (414). Furthermore, PTH actions on osteoblasts *in vitro* may be strongly influenced by their state of differentiation, and even opposite effects may appear as the cells differentiate (415, 416). In this regard, Tsuboi and Togari (417) described striking and opposite effects of hPTH(1–34) and hPTH(53–84) (10^{-7} M each) on alkaline phosphatase expression and dentine enamel formation in organ-cultured embryonic mouse tooth germ in which the opposing effects of the two hormone fragments were reversed at different stages of embryonic development.

Other biological effects of CPTH fragments not attributable to activation of the PTH1R have been reported in skeletal cell systems as well. Thus, Erdmann *et al.* (130) described selective activation of cytoplasmic Ca^{2+} transients by PTH(52–84) in isolated human fetal chondrocytes that occurred several minutes after addition of the peptide. Subsequent structure-function work from the same group narrowed the hPTH domain required for eliciting this effect to the Ala-Asp-Val-Asn sequence at residues 73–76, in that hPTH(52–84), hPTH(57–76), hPTH(61–80), and hPTH(64–84) all were active whereas hPTH(53–72) was not (418). These workers also observed that the increase in cytoplasmic Ca^{2+} concentrations in these chondrocytes was blocked by depletion of extracellular calcium but not by various inhibitors of intracellular calcium release; this suggested a mechanism involving augmented calcium influx that is triggered by activated CPTHRs but that likely requires one or more additional signaling events, given the relatively delayed nature of

the response. This conclusion is consistent with the previously described increased uptake of ^{45}Ca into SaOS-2 cells observed by Fukayama *et al.* (412). Zaman *et al.* (419) also observed that substitution of an aspartate for the native asparagine at position 76 of hPTH(1–84) greatly reduced bioactivity in cytochemical bioassays that measure glucose-6-phosphate dehydrogenase activity in either hypertrophic chondrocytes of rat metatarsals or distal convoluted tubular epithelia of guinea pig kidney slices. In fact, the Asp^{76}-substituted hPTH(1–84), as well as [Asp^{76}]hPTH(39–84), both functioned as antagonists of cytochemical bioactivity stimulated by native hPTH(1–84), hPTH(1–34), or cAMP, and these authors provided some evidence that the [Asp^{76}]hPTH may have exerted this antagonism by inducing phosphodiesterase activity (419). Because the extreme C terminus of PTH is not known to interact directly with PTH1Rs, the difference in bioactivity seen with Asp^{76}- *vs.* Asn^{76}-substituted PTH peptides may reflect differences in their interactions with CPTHRs, a conclusion supported by the similar findings with the [Asp^{76}]hPTH(39–84) peptide.

C. Actions of intact PTH and PTH C-terminal fragments in bone cells that lack PTH/PTHrP receptors

The studies of CPTH bioactivity described so far were performed in cells or tissues that endogenously express PTH1Rs. More direct evidence of bioactivity mediated by an independent class of CPTHRs was provided by experiments using PTH1R-null clonal cell lines. Thus, Divieti *et al.* (402) demonstrated that activation of CPTHRs in PTH1R-null osteocytes could affect cell survival and expression of the gap-junction protein connexin-43, thought to be important for intercellular communication among osteocytes and osteoblasts. In clonal conditionally immortalized PTH1R-null osteocyte-like cells already acclimated to nontransforming culture conditions for 5–7 d, hPTH(1–84), in a dose-dependent manner at concentrations ranging from 10^{-9} to 10^{-7} M, promoted apoptosis by up to 2-fold within 16 h of addition (Fig. 6) (402). This proapoptotic effect contrasts with the antiapoptotic response to PTH1R activation by hPTH(1–34) described in another clonal osteocyte-like cell line and in osteoblasts *in vitro* and *in vivo* (420). This proapoptotic effect in PTH1R-null clonal osteocytes was exerted not only by the full-length hormone, but also by hPTH(24–84) and by the shorter fragment PTH(39–84) (Fig. 6). In these cells, treatment for 2 h with PTH(1–84) (10^{-7} M) or PTH(39–84) (10^{-6} M) also induced an increase in connexin-43 staining that was especially prominent in a perinuclear location (402). Using clonal PTH1R-null osteoblastic and osteocytic cells, D'Ippolito *et al.* (421) also observed that hPTH(1–84), but not hPTH(1–34), could promote gap-junctional communication among these cells, as assessed by dye-transfer techniques.

D. Regulation of serum calcium and bone resorption by PTH C-terminal fragments

As noted earlier (see *Section IV.E.2*), novel two-site immunoassays for "intact PTH" now can distinguish certain long CPTH fragments from intact hPTH(1–84) *per se* (337, 338). These previously unrecognized extended CPTH fragments,

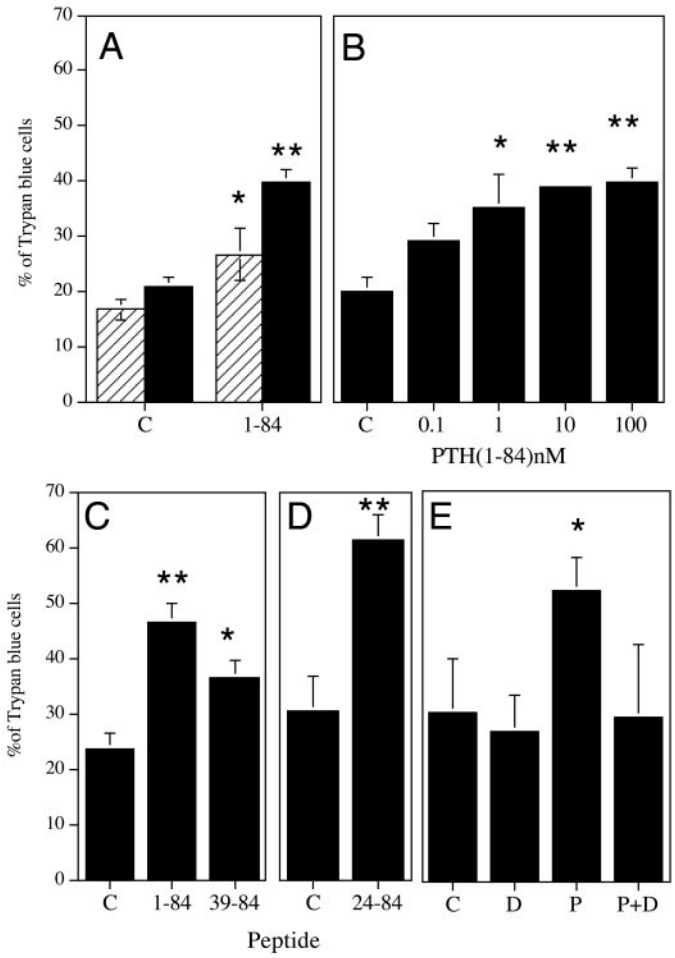

FIG. 6. Proapoptotic effect of CPTHR activation in clonal PTH1R-null osteocytes. Clonal OC59 cells were plated, cultured at 33 C for 3 d, and then incubated at 37 C (*hatched bars* in A) or 39 C (*solid bars*) for 5–7 d before addition of PTH peptides for an additional 16 h, followed by trypan blue staining. Because the cells are conditionally transformed with a temperature-sensitive SV40 T antigen that remains active at 33 C and partially so at 37 C, the higher temperature (39 C, *solid bars*) may permit greater rates of stimulated apoptosis. Responses shown are means ± SD (n = 4) of the percentage of trypan blue-positive cells for controls ("C") *vs.*: A, hPTH(1–84), 100 nM; B, hPTH(1–84) at indicated concentrations (nanomoles); C, hPTH(1–84) 100 nM, or hPTH(39–84) 1000 nM; D, hPTH(24–84), 100 nM; or E, 100 nM PTH(1–84) ("P") with or without the caspase-3 inhibitor peptide DEVD ("D") added 1 h earlier. *, $P < 0.05$; **, $P < 0.01$. [Reprinted with permission from P. Divieti *et al.*: Endocrinology 142:916–925, 2001 (402). © The Endocrine Society.]

indistinguishable from intact PTH by conventional two-site assays, behave chromatographically like hPTH(7–84) during reverse-phase HPLC (422). The actual chemical structures of these fragments have not been determined, but these observations prompted recent investigation of the possible biological actions of long N-truncated PTH fragments, for which the synthetic peptide hPTH(7–84) was selected as a model.

Thus, in 2000, Slatopolsky *et al.* (423) reported lowering of serum calcium over 2 h by ip administration of hPTH(7–84) (0.5 nmol every 30 min, or 2 nmol total) to hypocalcemic female rats that had been parathyroidectomized within the preceding 24 h and subsequently maintained on a low-cal-

cium diet. Moreover, this regimen of hPTH(7–84) treatment also blocked, almost completely and within 60 min, the rise in serum calcium otherwise induced by hPTH(1–84) when this peptide was coadministered at an equimolar dose (Fig. 7). In the same study, the phosphaturic effect of an 80-min infusion of hPTH(1–84) was reduced approximately 50% by administration of a 4-fold molar excess of hPTH(7–84). Nguyen-Yamamoto *et al.* (424) subsequently reported similar results in acutely thyroparathyroidectomized male rats, except that the animals were studied only 2 h after parathyroidectomy, had been maintained on a normal diet and received the PTH peptides via continuous iv infusion rather than as evenly spaced, ip bolus injections. In this study, PTH(7–84), infused at 10 nmol/h for 2 h, also reduced serum calcium (to 16% below controls) and blocked the calcemic response to hPTH(1–34) or hPTH(1–84) administered at 1 nmol/h. Interestingly, this anticalcemic effect was seen also with 10 nmol/h of a mixture of CPTH fragments, composed of 45% hPTH(39–84), 45% hPTH(53–84), and 10% hPTH(7–84), but only when coinfused with hPTH(1–34). The CPTH fragment mixture slightly potentiated the effect of hPTH(1–84), a response interpreted as consistent with competition by the CPTH fragments for CPTHR-mediated binding or enzymatic clearance of the intact hormone. Less potent antagonism of the hPTH(1–34) response by 3 nmol/h of hPTH(7–84), compared with 10 nmol/h of CPTH mixture, pointed also to a biological effect of the shorter CPTH fragments, although these clearly were less potent than hPTH(7–84). The infused CPTH fragments (unlike the N-intact peptides) also lowered serum phosphate slightly. Because the renal excretion of neither phosphate nor calcium was increased in a sustained manner by these fragments, the results were most consistent with decreased fluxes of these ions out of bone. Experiments with rat osteosarcoma cells *in vitro* showed that the hPTH(7–84) could not bind to PTH1Rs even at concentrations 100-fold higher than those required for displacement by PTH(1–34) or PTH(1–84), nor did it antagonize the cAMP response to hPTH (424). The hPTH(7–84) did bind to CPTHRs on these cells, however, as illustrated using ^{125}I-[Tyr^{34}]hPTH(19–84) radioligand (424). In a preliminary report, Faugere *et al.* (425) noted that coadministration of hPTH(7–84) by continuous infusion for 2 wk to thyroparathyroidectomized rats with chronic renal failure antagonized the increase in bone turnover, as well as in serum calcium levels, otherwise produced by continuously infused PTH(1–84).

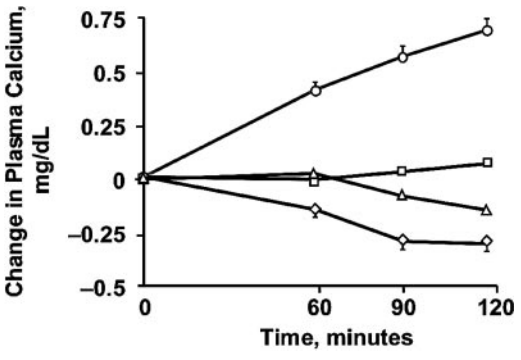

FIG. 7. Comparison of the calcemic effects of PTH isoforms. Parathyroidectomized rats fed a 0.02% calcium diet show a significant increase in plasma calcium after treatment with hPTH(1–84). In contrast, hPTH(7–84) produced a slight but significant decrease in plasma calcium. When both peptides were given together in a 1:1 molar ratio, the calcemic response induced by hPTH was reduced by 94% ($P < 0.001$). ○, hPTH(1–84), n = 9; □, hPTH(1–84) + hPTH(7–84), n = 6; △, control, n = 5; ◇, hPTH(7–84), n = 5. [Reprinted with permission from E. Slatopolsky *et al.*: Kidney Int 58:753–761, 2000 (423). © The International Society of Nephrology.]

These important *in vivo* experiments indicate that large N-truncated PTH fragments such as PTH(7–84) can exert hypocalcemic effects and also effectively antagonize the calcemic response to PTH1R activation, at least in parathyroidectomized animals. Such effects are not seen with PTH(3–84), a more effective PTH1R antagonist than PTH(7–84) (424, 426–428), which suggests that they likely result from actions at receptors different from the PTH1R. This is supported by the apparent bioactivity of the shorter CPTH fragments hPTH(39–84) and hPTH(53–84), which cannot bind to PTH1Rs and, when coinfused with PTH(1–84), actually accentuated rather than inhibited the PTH1R-mediated response (424). Furthermore, the fact that the anticalcemic effect occurs in the absence of a calciuric response and is seen in animals on a low-calcium diet strongly points to bone as the source of the calcium and, thus, the target of CPTH action, in these experiments.

The possibility that CPTH fragments may act directly on bone to inhibit bone resorption was addressed by measuring the liberation of previously incorporated radiocalcium from neonatal murine calvarial bones over 72 h in organ culture (428). This work showed that hPTH(7–84) lowered the basal rate of bone resorption as effectively as calcitonin and strongly inhibited accelerated resorption induced by any of several agonists, including both PTH(1–34) and PTH(1–84) but also others that act independently of the PTH1R [1,25-$(OH)_2D_3$, prostaglandin E_2, and IL-11] (Fig. 8). These effects were dose-dependent and required relatively high concentrations of hPTH(7–84) (100–300 nM), although the bioavailability of the peptide over several days in the *in vitro* system was not addressed. Importantly, the antiresorptive effect was not mimicked by peptides such as hPTH(3–34)NH_2 or [Leu^{11}, D-Trp^{12}, Trp^{23}]hPTHrP(7–36)NH_2, both of which are effective PTH1R antagonists [whereas hPTH(7–84) is not] (428). The hPTH(7–84) peptide, but not the PTH1R antagonists, also inhibited formation of osteoclasts induced by 1,25-$(OH)_2D_3$ in 12-d cultures of normal murine bone marrow. In subsequent work, this antiosteoclastogenic effect of hPTH(7–84) was observed using receptor activator of nuclear factor-κB ligand/macrophage colony-stimulating factor-stimulated purified hematopoietic osteoclast precursors, which were found to exhibit specific binding of the CPTH radioligand, ^{125}I-[Tyr^{36}]hPTH(19–84), but not of the ^{125}I-rat PTH(1–34) PTH1R radioligand (429). These findings are compatible with earlier reports of the binding of intact ^{125}I-PTH to cells of the osteoclast lineage (352–354), as described above (see *Section V.B*). Although the mechanism of the anticalcemic effect of PTH(7–84) *in vivo* remains uncertain, it seems that CPTH fragments, acting directly through CPTHRs expressed on osteoclast precursors of the hematopoietic lineage and possibly also on marrow stromal cells that support osteoclast formation and activity (Fig. 4C), can inhibit bone

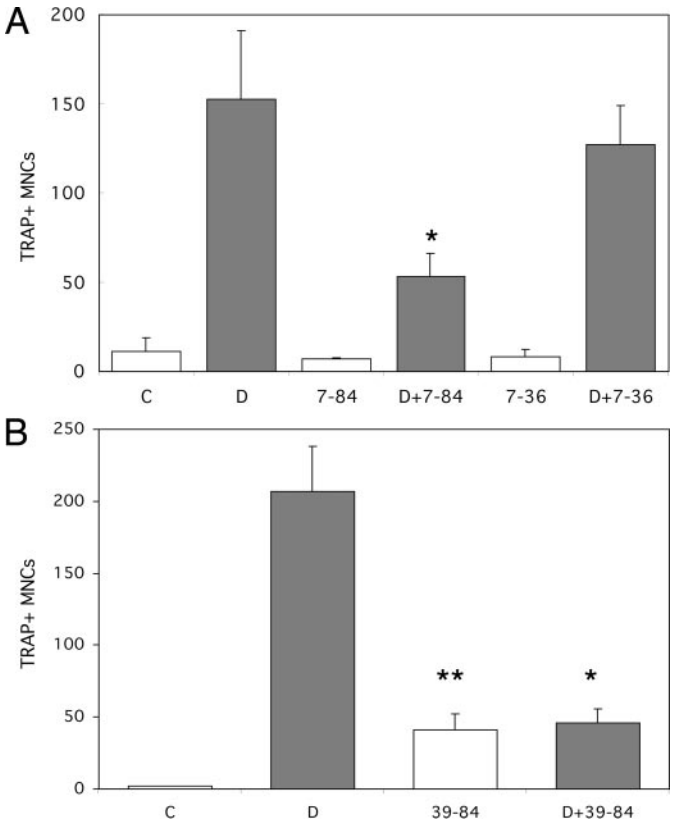

FIG. 8. Inhibition of osteoclast generation by hPTH(7–84) and hPTH(39–84). Normal murine bone marrow cells were cultured for 12 d, indicated agents were added three times weekly, and cells then were fixed and stained for tartrate-resistant acid phosphatase (TRAP). *Bars* depict mean ± SD of the numbers of TRAP-positive cells with three or more nuclei in each group (n = 3 wells/group). Cultures were treated with vehicle alone ("C") or with indicated combinations of 1,25-$(OH)_2D_3$ ("D"), 10 nM; hPTH(7–84), 300 nM; hPTHrP(7–36) analog, 300 nM; or hPTH(39–84), 3000 nM. *, $P < 0.05$ *vs.* D alone. **, $P < 0.05$ *vs.* control. [Reprinted with permission from P. Divieti *et al.*: Endocrinology 143:171–176, 2002 (428). © The Endocrine Society.]

resorption, at least in part by reducing the rate of formation of new osteoclasts.

One paradoxical aspect of these *in vitro* analyses was the finding that whereas shorter CPTH peptides such as hPTH(39–84) also can inhibit osteoclast formation induced in whole bone marrow cultures by 1,25-$(OH)_2D_3$, they exert weak stimulation of osteoclast formation when added alone, in the absence of stronger agonists (428). Such dichotomous effects were not seen with hPTH(7–84), which caused no osteoclast formation when added alone. A weak agonist action also was observed with hPTH(53–84) in a coculture system in which clonal, conditionally immortalized MS1 murine marrow stromal cells support osteoclast formation from precursors present among normal murine spleen cells (406, 430). Stimulation of osteoclast formation by CPTH fragments [hPTH(35–84) > hPTH(53–84) > hPTH(69–84)], introduced at concentrations of 1 and 10 nM, also was reported previously in 7-d cultures of mixed bone cells isolated from long bones of young mice (431). Osteoclasts could be induced by these CPTH peptides as well within 4 d of their addition to cultures of GM-CSF-dependent splenic hematopoietic blast cells or by incubation of untreated osteoclast progenitors in conditioned medium collected from UMR-106 rat osteosarcoma cells that previously had been exposed to these fragments (431). Interestingly, whereas both hPTH(1–34) and hPTH(1–84) were powerful agonists in these *in vitro* systems, hPTH(1–84) was significantly more potent than hPTH(1–34) at higher concentrations (10 nM), which was felt to be consistent with the possibility that hPTH(1–84) might access CPTHRs not available to hPTH(1–34). These investigators also showed that neither hPTH(1–34), hPTH(1–84), nor CPTH fragments exerted any effect on survival of, or bone resorption by, isolated mature osteoclasts (431).

Thus, the available evidence concerning actions of CPTH fragments on osteoclast formation and bone resorption supports a complex scenario in which CPTHRs may be expressed by both osteoblastic stromal cells of mesenchymal origin and by osteoclast progenitors or precursors of the hematopoietic lineage. Moreover, there appear to be length-dependent differences in bioactivity among different CPTH fragments, in that hPTH(7–84) acts only to inhibit osteoclast formation, whereas shorter peptides such as PTH(39–84) and PTH(53–84) both can antagonize stronger agonists and also exert a (weak) intrinsic agonist effect. Whether these disparate responses reflect actions at CPTHRs coupled to different intracellular effector mechanisms in different target cells (*i.e.*, marrow stromal *vs.* hematopoeitic cells) or different patterns of signaling elicited via one class of CPTHRs on a single target cell type, or both, remains to be established.

VIII. Summary of Evidence for Distinct Receptors for the C Terminus of PTH

Available evidence supporting the existence of an independent class of receptors with specificity for the C-terminal region of PTH is now compelling. Analysis of radioligand binding to these CPTHRs has evolved from the initial recognition that radiolabeled PTH(1–84) can access renal and osseous binding sites unavailable to PTH(1–34), through the finding that modified N-truncated PTH radioligands incapable of effectively interacting with PTH1Rs nevertheless can exhibit specific binding to osteosarcoma cells, to the definitive demonstration of identical specific binding of such a modified radioligand to clonal bone cells that genetically lack PTH1Rs. Observations of similar patterns of ligand selectivity of these binding sites among diverse cell lines provide additional evidence for expression of similar or identical species of CPTHRs. These demonstrations of CPTH-specific binding sites have been accompanied by a growing body of evidence showing that peptides capable of interacting with these sites also can exert unique biological actions on cells and tissues of skeletal origin (Tables 1 and 2). Early evidence of differences in biological activity between PTH and the N-terminal fragment PTH(1–34), both full agonists for the PTH1R (Table 1), was followed by direct demonstrations of unique actions on bone cells of hormone fragments comprising portions of the C terminus that cannot bind or activate PTH1Rs (Table 2). The finding that such CPTH fragments, as well as intact PTH, can regulate the behavior of clonal osteocytes that genetically lack PTH1Rs provides par-

TABLE 1. Systems in which biological responses to intact PTH differ from those to PTH(1–34)

Biological response	Experimental system	PTH(1–34)	PTH(1–84)	First author (ref.)
Gluconeogenesis	Normal dogs	NE	++	Hruska (369)
Natriuresis and calciuria	TPTX dogs	++	NE	Puschett (370)
cAMP production	Perfused canine tibia	++	NE	Martin (371)
Erythroid burst formation	Murine bone marrow	NE	−−	Meytes (372)
Leukocyte migration, elastase release	Rat peripheral blood cells	NE	++	Doherty (373); Massry (374)
Cytosolic free Ca^{2+}	Rat thymocytes, adipocytes	NE	++	Stojceva-Taneva (375); Ni (376)
Volume of pancreatic secretions	Rats	NE	++	el-Shahawy (377)
Rate of beating	Rat cardiomyocytes	++	+++	Bogin (378)
Cytosolic free Ca^{2+}	Rat islets, cardiomyocytes, hepatocytes, renal cells	++	+++	Fadda (379); Smogorzewski (380); Klin (381); Tanaka (382)
Proliferation	Human B cells	++	+++	Alexiewicz (383)
Serum fibronectin and 1,25-$(OH)_2D$	Rats	+	+++	Sun (384)
Secreted fibronectin	ROS 17/2.8 cells	+	+++	Sun (384)
Osteoclast formation	Mixed long bone cells, splenic blast cells	++	+++	Kaji (431)

+, Stimulation; −, inhibition; NE, no significant effect observed; TPTX, thyroparathyroidectomized.

ticularly strong evidence for the presence in these cells of CPTHRs that can operate independently of the PTH1R. At the same time, convincing evidence for *in vivo* anticalcemic actions of hPTH(7–84) and of other shorter CPTH fragments incapable of activating PTH1Rs, together with the finding that such peptides also can exert antiresorptive and antiosteoclastogenic effects *in vitro*, further suggests the presence of functional CPTHRs in bone with potentially important physiological roles, although part of these effects could be accounted for by PTH1R down-regulation induced by CPTH fragments, as mentioned above in *Section III.B* (219). More work clearly is required, but a strong case now can be made that functional CPTHRs exist on the surfaces of many cell types in bone and that these receptors may be involved in mediating novel regulatory actions of PTH.

IX. Biological, Pharmacological, and Clinical Implications of Current Knowledge

Appreciation of the existence of CPTHRs on bone cells, together with the presence of their potential ligands in the form(s) of circulating CPTH fragments, has opened a new window into the physiology of PTH and the pathophysiology of disorders that involve abnormalities in PTH secretion, peripheral metabolism and clearance, or both. The earlier misconception that CPTH fragments are "biologically inactive", which is true only with respect to activation of classical PTH1Rs, has been supplanted by the recognition that peptides comprising the C-terminal portion of PTH can exert unique biological effects in appropriately selected bioassays, both *in vitro* and *in vivo*. The potential physiological role of the CPTH/CPTHR system can only be dimly perceived at present, but important clues are accumulating, and some speculation is appropriate.

It has been convincingly shown that parathyroid gland secretion of PTH is stimulated by hypocalcemia, whereas secretion of CPTH fragments is promoted by hypercalcemia (see *Section IV.D*). A metabolic schema depicting the regulated production of PTH and CPTH fragments and their interaction with PTH1Rs and CPTHRs in bone is presented in Fig. 9. Evidence that parathyroidal secretion of CPTH fragments is favored by hypercalcemia and that certain CPTH fragments inhibit osteoclast formation and bone resorption via direct effects on cells of the hematopoietic lineage suggests the possibility of a negative feedback loop that could serve physiologically to restrain release of calcium from bone into blood when it is not needed. Thus, CPTHRs may mediate a protective effect on the skeleton, by limiting bone resorption and tipping the balance in favor of net bone formation, during states of relative hypercalcemia, or even normocalcemia, when CPTH levels greatly exceed those of intact PTH. In this respect, the CPTH/CPTHR system may be seen as antagonistic to the actions of N-terminal PTH via the PTH1R, which increases osteoclastic activity in response to hypocalcemia. In addition to reducing hypercalcemia by slowing bone resorption, CPTH secreted by the parathyroid could also be postulated to play a physiological role as a bifunctional regulator of skeletal calcium stores. Thus, the parathyroids could operate to replete skeletal calcium stores when serum calcium concentration is normal or high during times of adequate calcium availability, via CPTHR restraint of bone resorption, but to call on them via predominant PTH1R activation when dietary calcium or vitamin D availability is restricted, as sensed by a downward trend in extracellular calcium concentrations via the parathyroid calcium-sensing receptor.

Such functional antagonism between PTH1Rs and CPTHRs has been observed as well for several other responses in cells of the osteoblastic lineage, including regulation of alkaline phosphatase, procollagen I, and apoptosis (Table 2). The proapoptotic response of clonal osteocytes, which has been observed also in marrow stromal cells and osteoblasts (P. Divieti, unpublished observations), could reflect a more general role for CPTHRs in limiting the life span of cells in the osteoblastic lineage and in modulating the antiapoptotic effect of PTH1R activation reported in such cells.

The extraordinarily high density of CPTHRs on osteocytes, by far the most abundant of bone cells, is of particular interest. This finding, coupled with preliminary evidence that

TABLE 2. Biological responses to PTH, PTH(1–34), and C-terminal PTH fragments

Biological response	Experimental system	PTH(1–34)	PTH(1–84)	CPTH fragments	First author (ref.)
Transient activation of G6PD	Guinea pig renal slices	Early		Late	Arber (395)
Alkaline phosphatase regulation	ROS 17/2.8 cells	−−	−−−	++	Murray (407, 408); Nakamoto (411); Takasu (399)
Osteocalcin gene expression	ROS 17/2.8 cells	NE		++	Kung-Sutherland (410)
Collagen 1 gene expression	ROS 17/2.8 cells	++		NE	Kung-Sutherland (410)
Osteocalcin gene expression	UMR 106-01 cells	−−	NE	++	Nasu (413)
IGFBP-5 gene expression	UMR 106-01 cells	++	++	++	Nasu (413)
^{45}Ca influx	SaOS-2 cells	++		++	Fukayama (412)
Cytosolic free Ca^{2+}	Fetal chondrocytes	++		++	Erdmann (130)
Collagen type II and X gene expression	Fetal chondrocytes	++		++	Erdmann (130)
Osteoclast formation	Whole murine marrow	++		+	Liu (406)
Alkaline phosphatase and enamel formation	Embryonic tooth germ	++		−−	Tsuboi (417)
	Early stage				
	Late stage	−−		++	
Connexin 43 expression	PTH1R-null osteocytes	NE	++	++	Divieti (402)
Apoptosis	PTH1R-null osteocytes	NE	++	++	Divieti (402)
Apoptosis	Clonal PTH1R+ osteocytes	−−			Jilka (420)
Dye transfer via gap junctions	PTH1R-null osteocytes	NE	++		D'Ippolito (421)
Serum calcium	TPTX rats-controls		++	−	Slatopolsky (423)
	Increase due to PTH(1–84)			−−	
Serum calcium	TPTX rats-controls	++	++	−	
	Increase due to PTH(1–34)			−−	Nguyen-Yamamoto (424)
Serum calcium and bone turnover	TPTX rats with renal failure		++		Faugere (425)
	Increase due to PTH(1–84)			−−	
^{45}Ca release from bone	Mouse calvariae *in vitro*	++	++	−	Divieti (428)
Osteoclast formation	Mixed long bone cells	++	+++	++	Kaji (431)
	Splenic blast cells	++	+++	++	
Osteoclast formation	Whole murine marrow, agonist-stimulated			−−	Divieti (428)
	Basal			−−	
Stimulated osteoclast formation	Purified osteoclast precursors	NE		−−	Divieti (429)

+, Stimulation; −, inhibition; NE, no significant effect observed; blank cell, not tested; G6PD, glucose-6-phosphate dehydrogenase; TPTX, thyroparathyroidectomized.

CPTHRs regulate gap-junctional communication, could place the CPTH/CPTHR system at center stage in the crucial mechanosensory function of the osteocyte network. It also seems possible that osteocytes (which normally express PTH1Rs) may be involved, at least in part, in mediating the acute calcemic response to PTH observed in parathyroidectomized rats *in vivo*. The mechanism involved could represent a form of osteocytic osteolysis, which has been suggested on the basis of very rapid compatible ultrastructural changes in bone after PTH administration (432, 433). Indeed, the antagonism of the PTH calcemic response by PTH(7–84) occurs so quickly that it seems unlikely to be explained entirely by inhibition of osteoclast formation alone, and mature osteoclasts occupy a very limited surface of bone. Thus, it may be appropriate to consider further the possibility that CPTHRs could diminish the stimulation of local osteolysis by PTH1Rs.

Although more information is needed, experiments to date indicate that CPTHRs do not recognize sequences within PTHrP. This could provide target cells with a means to discriminate between PTH and PTHrP acting on adjacent PTH1Rs. Because the affinity of CPTHRs for PTH is at least 10-fold lower than that of PTH1Rs, however, the efficiency of a CPTHR-dependent parallel signal may be limited when PTH interacts with both the PTH1R and the CPTHR. On the other hand, CPTH fragments circulate at molar concentrations that exceed those of PTH by at least 3- to 10-fold, and perhaps more, depending on renal function and the ambient serum calcium concentration (see *Section IV.D*). Moreover, CPTH fragments longer than PTH(24–84) bind to CPTHRs with the same affinity as PTH. Thus, it seems likely that circulating CPTH fragments, whether secreted by the glands or generated by hepatic metabolism of PTH, are the primary physiological ligands for CPTHRs. Furthermore, as reviewed in *Sections IV and V*, CPTH fragments of different lengths exist in blood and may exhibit substantial differences in CPTHR binding affinity and in biological actions attributed to CPTHR activation. This raises the possibility that the rate at which specific CPTH fragments of various lengths are generated could be regulated physiologically to adjust the availability of active CPTHR ligands in the bloodstream.

Better understanding of the roles of the CPTH/CPTHR system in bone biology and calcium metabolism is especially timely in light of the fact that PTH peptides currently are being introduced as therapeutics for osteoporosis and, potentially, other disorders of bone. Moreover, calcimimetics

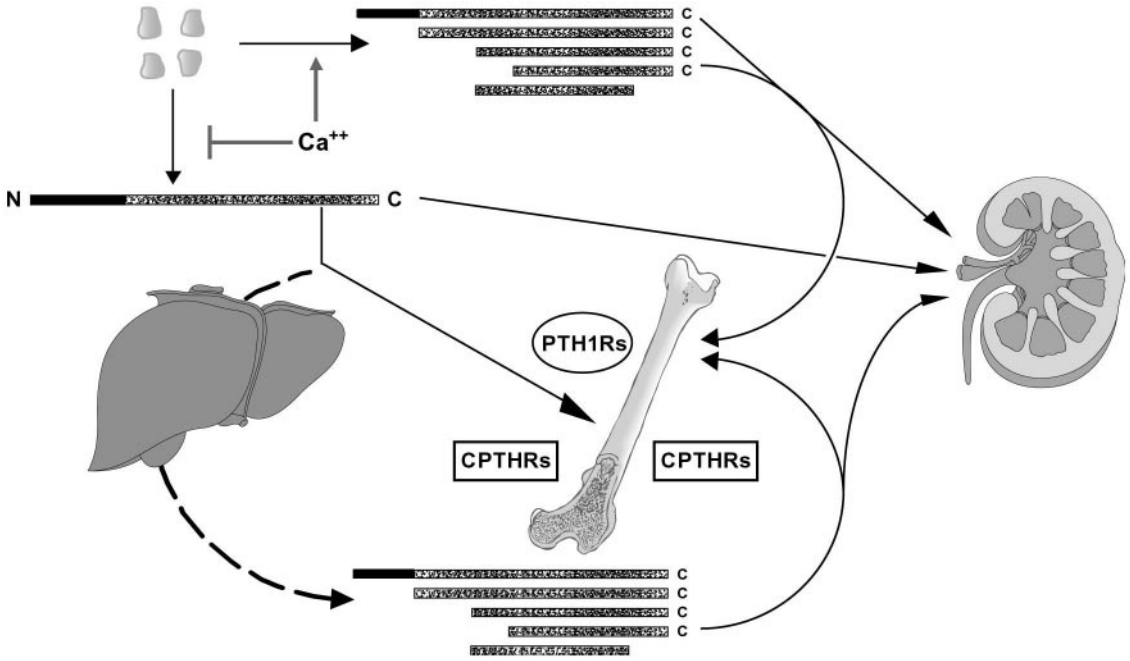

FIG. 9. Secretion and metabolism of PTH. Secretion of intact PTH by the parathyroid glands is accompanied by release of a family of CPTH fragments (*stippled shading*), the amount of which, relative to intact PTH, increases at higher serum calcium concentrations. Intact PTH acts directly on PTH1Rs via its N-terminal sequence (*black shading*) in target tissues (*i.e.*, bone) or is directly cleared by liver or kidney. Hepatic clearance of PTH involves rapid proteolysis by Kupffer cells to N-terminal fragments (degraded *in situ*) and a series of C-terminal fragments, some of which reappear in the circulation (see *Sections IV.B* and *IV.C*). CPTH fragments produced by the parathyroids and the liver are chemically similar or identical and include minimally N-truncated, long forms and some midregion fragments truncated at both ends. Like intact PTH, CPTH fragments can exert direct effects on bone cells via a novel class of CPTH receptors. CPTH fragments normally circulate at 5-fold higher concentrations than intact PTH but are cleared predominantly by the kidney and thus accumulate disproportionately during renal failure.

and calcilytics, small molecules developed as agonists and antagonists, respectively, of the parathyroid calcium-sensing receptor, also are undergoing clinical trials to assess their utility in managing primary or secondary hyperparathyroidism (calcimimetics) (434–436) or as surrogates for injectable PTH in osteoporosis therapy (calcilytics) (435, 437, 438). Given that CPTH fragments arise both by direct glandular secretion (in a calcium-sensitive manner) and by peripheral proteolysis of intact hormone (the role of calcium in which is unsettled), effects of these therapeutics on levels of specific CPTH fragments in blood could become important in their actions on bone. For example, administration of PTH(1–34) or a calcimimetic agent would be expected to reduce endogenous parathyroid secretion and, as well, the absolute rate at which CPTH fragments are generated via secretion or hepatic proteolysis of intact PTH. Administration of PTH(1–84) or a calcilytic, on the other hand, could engender higher levels of circulating CPTH fragments produced by peripheral metabolism. In such scenarios, individual differences in CPTH regulation, as well as the functional interactions between PTH1Rs and CPTHRs in bone, could prove to be important predictors of the therapeutic result.

Another important clinical implication of the new knowledge concerning circulating CPTH fragments and their receptors relates to the use of immunometric assays to assess serum PTH levels in patients with primary or secondary hyperparathyroidism and in those with chronic renal disease. With recognition that so-called first-generation immunometric assays may detect not only intact PTH but also long CPTH fragments, it now is clear that much of the signal interpreted hitherto as intact PTH, especially in uremia, is in fact due to CPTH fragments that may well have biological activities that are functionally opposite those of the intact hormone, as it acts via PTH1Rs. This certainly could explain why attempts to suppress intact PTH into the normal range with vitamin D and calcium therapy may be accompanied by low turnover or aplastic bone disease (439, 440). It is likely that patients with primary or secondary hyperparathyroidism will be found to exhibit considerable individual variation with respect to the relative amounts of intact hormone and CPTH fragments in their blood (338). Whether, as suggested by some early reports (340), this will translate into improved assessment of skeletal status through use of second-generation immunometric assays to distinguish PTH from long CPTH fragments remains to be seen. Complete understanding of the significance of these long circulating CPTH fragments in human disease must await more information concerning their biochemical structure(s) and possible biological activities.

X. Directions for Future Research

Many questions remain to be answered in future research. Perhaps the most immediate questions relate to our knowledge of the structure of the C-terminal receptors, which is rudimentary, and our knowledge of the signaling mecha-

nisms of the receptors, which so far is almost nonexistent; the only intracellular signaling responses identified to date have been changes in cytosolic calcium (in chondrocytes) and in calcium uptake (osteosarcoma cells) (see *Section VII.B*). As reviewed in *Section III.A*, CPTH fragments do not activate adenylyl cyclase, so CPTHRs presumably are not coupled to adenylate cyclase.

The agenda for research in this field is indeed full and unquestionably will be greatly accelerated by the molecular cloning of cDNA(s) encoding the CPTHR(s), efforts toward which are well under way at present. Such an advance would enormously facilitate analysis of the pharmacology and cell- and tissue-specific expression of these receptors, the possibility that they exist in multiple forms, their likely modes of signal transduction, and their associated patterns of regulation of gene expression in target cells. A potent probe of their role in normal physiology will be the use of gene-ablation technology to eliminate CPTHR expression in selected target tissues, to determine the relative importance of the CPTHRs *vs.* PTH1R down-regulation in mediating the antiresorptive and hypocalcemic effects of CPTH fragments. More work is needed to define the cellular actions of CPTHRs *in vitro* and *in vivo*, particularly in bone, and to define the actions of CPTHRs on bone remodeling *in vivo*. CPTHR effects also need to be investigated further in renal epithelial cells where they first were identified. Evidence from *in vitro* systems that CPTHRs are expressed at much higher levels in osteocytes than in other cells of the osteoblastic lineage must be confirmed *in vivo*, and the likely special role these receptors play in osteocyte biology must be elucidated further. A fuller understanding of the biological responses to CPTHR activation is required, in osteoblastic cells as well as in osteoclasts and their progenitors, to enable more effective exploration of the apparent complexity of bioactivities among various CPTH ligands already glimpsed in the early experiments reviewed here. With respect to these ligands, the enormous current void in knowledge of the precise structures of circulating conventional, mid, late, and long CPTH fragments must be filled before it will be possible to accurately measure blood levels of these fragments or to assess their specific activities at CPTHRs. Additional work must be done to ascertain the extent to which individual CPTH fragments in blood arise from glandular secretion *vs.* peripheral proteolysis of intact hormone and whether the distribution of biochemically defined fragments derived from either source is controlled by extracellular calcium or other physiological regulators. The fact that CPTHRs are expressed on cells of both the osteoclast and osteoblast lineages, exhibit striking ligand specificity, and may be capable of transducing diverse functional responses in a highly ligand-selective manner makes them potentially attractive therapeutic targets in the management of bone disease. Accordingly, more basic efforts directed at understanding the molecular events initiated by CPTHR activation in appropriate target cells, including specific signaling events and regulated genes, and the possibility of modulating these responses through ligand design should be an important priority as well.

Clinical research must focus on the development and utilization of new assays to assess the contributions of different circulating forms of PTH to the heterogeneity of circulating PTH. This may have significance in assessing skeletal status and predicting responses to novel therapies for bone or parathyroid disease, including PTH itself. The expectation that CPTHR activation could modulate the response to calcilytic agents or intermittently administered PTH(1–84), as compared with PTH(1–34), should be tested using careful measures of bone quality and microarchitecture together with specific assays of generated CPTH fragments. Although clearly a strong possibility, it is not known whether high circulating CPTH fragments contribute to any form(s) of renal osteodystrophy. Interest in these issues should fuel efforts to better define circulating PTH peptides, using available highly sensitive analytical techniques to assess the potential utility of CPTHR antagonists in this setting.

The possibility that the proapoptotic effect of CPTHRs, as seen in osteocytes, might afford protection against the development of skeletal neoplasms, as observed in rat toxicology studies with PTH(1–34) (214), is of particular interest and should be assessed. In clinical trials of hPTH(1–84) therapy of osteoporosis, concomitant activation of PTH1R and CPTHRs by PTH(1–84) might, via inhibition of bone resorption, modify dose-response curves, or modify side effects such as hypercalcemia or hypercalciuria. These questions are being explored by clinical trials currently in progress (368).

Clearly, this rapidly evolving field presents many opportunities for future research at both the fundamental and clinical level. In view of the finding of regulated secretion of both intact PTH and CPTH fragments by the parathyroids, it will be necessary, particularly when interpreting physiology, to consider the involvement of CPTH fragments, together with PTH itself, as potential contributors to regulation of calcium and bone metabolism.

Acknowledgments

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