Amino-Terminal Modifications of Human Parathyroid Hormone (PTH) Selectively Alter Phospholipase C Signaling via the Type 1 PTH Receptor: Implications for Design of Signal-Specific PTH Ligands†

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ABSTRACT: Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) activate the PTH/PTHrP receptor to trigger parallel increases in adenylyl cyclase (AC) and phospholipase C (PLC). The amino (N)-terminal region of PTH-(1−34) is essential for AC activation. Ligand domains required for activation of PLC, PKC, and other effectors have been less well-defined, although some studies in rodent systems have identified a core region [hPTH-(29−32)] involved in PKC activation. To determine the critical ligand domain(s) for PLC activation, a series of truncated hPTH-(1−34) analogues were assessed using LLC-PK1 cells that stably express abundant transfected human or rat PTH/PTHrP receptors. Phospholipase C signaling and ligand-binding affinity were reduced by carboxyl (C)-terminal truncation of hPTH-(1−34) but were coordinately restored when a binding-enhancing substitution ($Glu^{19} \rightarrow Arg^{19}$) was placed within hPTH-(1−28), the shortest hPTH peptide that could fully activate both AC and PLC. Phospholipase C, but not AC, activity was reduced by substituting Gly^1 for Ser^1 in hPTH-(1−34) and was eliminated entirely by removing either residue 1 or the α-amino group alone. These changes did not alter binding affinity. These findings led to design of an analogue, [Gly^1,Arg^{19}]hPTH-(1−28), that was markedly signal-selective, with full AC but no PLC activity. Thus, the extreme N-terminus of hPTH constitutes a critical activation domain for coupling to PLC. The C-terminal region, especially hPTH-(28−31), contributes to PLC activation through effects upon receptor binding but is not required for full PLC activation. The N-terminal determinants of AC and PLC activation in hPTH-(1−34) overlap but are not identical, as subtle modifications in this region may dissociate activation of these two effectors. The [Gly^1,Arg^{19}]hPTH-(1−28) analogue, in particular, should prove useful in dissociating AC- from PLC-dependent actions of PTH.

Full understanding of the complex biological roles of parathyroid hormone (PTH)[1] and efforts to utilize its therapeutic potential require dissection of the multiple signaling patterns and cellular pathways of action of the hormone. PTH binds and activates specific receptors in renal and osseous target cells that also recognize PTH-related peptide (PTHrP) (*1*). In renal and osteoblastic cell lines, PTH triggers several parallel intracellular signaling responses, including activation of adenylyl cyclase (AC), protein kinase A (PKA), phospholipase C (PLC), and protein kinase C (PKC) and generation of second messengers such as cyclic AMP (cAMP), inositol trisphosphate (IP_3), diacylglycerol, and increased cytosolic free calcium (Ca_i^{2+}) (*2*−*16*). To date, two structurally related but distinct species of PTH receptors have been cloned (*2*, *17*, *18*). The first of these, type 1, was isolated from both bone and kidney cells and shown to transduce multiple signaling responses to PTH-(1−34) or PTHrP(1−36) when heterologously expressed in cells that lack endogenous type 1 PTH/PTHrP receptors (PTHRs) (*2*−*4*, *10*, *14*, *19*, *20*).

The relative efficiency with which activated PTHRs couple to AC vs PLC differs and is influenced strongly by the nature of the target cell and especially by the level of receptor expression on the cell surface. In transfected COS-7, HEK-293, or LLC-PK1 cells, for example, half-maximal coupling to AC/PKA occurs at ligand concentrations 10−30-fold lower than those required for activation of PLC, whereas PLC activation is much more sensitive to increases in receptor expression (*10*, *21*−*23*). The density of PTHRs on the surface of specific normal target cells in relevant tissues in vivo is not known with certainty but may lie within the range (i.e., 100000−500000 per cell) over which PLC is efficiently activated in these in vitro model systems (*24*, *25*).

With respect to the PTH ligand, previous efforts to define the contributions of specific regions of PTH to its binding and signaling properties have been undertaken mainly by

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[1] Abbreviations: AC, adenylyl cyclase; C, carboxyl; N, amino; Ca_i^{2+}, cytosolic free calcium concentration; cAMP, cyclic adenosine-3′,5′-monophosphate; EC_{50}, concentration required for 50% of maximal effect; FBS, fetal bovine serum; IBMX, isobutylmethylxanthine; IC_{50}, concentration required for 50% inhibition of specific binding; IP_3, inositol 1,4,5-trisphosphate; NMR, nuclear magnetic resonance; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; PTHR, type 1 PTH/PTHrP receptor.

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use of complex in vivo bioassays, organ cultures, isolated cell membranes or cell lines, generally of rodent origin, that may express more than one type of endogenous PTH receptors (*11, 16, 26*−*31*). Early structure/function studies of bovine PTH-(1−34), performed with isolated renal membranes, identified the key role of the carboxyl (C)-terminal bPTH-(25−34) region for receptor binding and of the amino (N)-terminus (i.e., Ala^1) for AC activation (*28, 29*). Later work conducted in vitro with intact renal tubules or with cultured renal or bone cells, however, indicated that N-truncated analogues such as PTH-(3−34), although unable to stimulate AC, could fully activate PKC and could regulate certain PKC-dependent distal biologic responses (*11, 16, 27*). Amino-truncated analogues of PTH-(1−34) also were found to increase PLC activity or Ca_i^{2+} in some cells (*6, 8, 16*) though not in others (*32, 33*). Studies of the signaling properties of the cloned type 1 PTHR have focused almost exclusively upon activation of AC, PLC or Ca_i^{2+} (*2, 4, 10, 14, 19, 20*), although stimulation of PKC and of PKC-dependent ion transport by hPTH-(1−34), hPTH-(3−34), and other hPTH fragments was reported in CHO cells transfected with rat PTHR cDNA (*3*). Collectively, these observations have engendered the concept that the structural determinants for activation of AC/PKA signaling are distinct from those required for activation of PLC or PKC and that these reside, respectively, within the N- and C-terminal domains of PTH-(1−34) (*12, 29, 34*). In particular, the region hPTH-(29−32) was identified specifically as a critical PKC activation domain (*12, 34*).

Compared with what is known from these studies of the rat PTHR, much less information is available regarding the structural features of human PTH required for binding to the human PTHR or for activation of its various signaling modes. Alanine-scanning mutagenesis has highlighted the importance of the C-terminal portion of hPTH-(1−34) for binding to the rat PTHR (*35*). Functional studies of transfected human PTHRs in COS-7 or HEK 293 cells have confirmed that hPTH-(1−34) activates AC and Ca_i^{2+}, although stimulation of PLC was not observed consistently and responses that were reported were modest (*14, 15, 19, 36*). The effects of hPTH-(3−34) on Ca_i^{2+} are similarly controversial (*14, 19*), while the roles of other regions of the hPTH-(1−34) molecule in signaling via the human PTHR have not been systematically addressed. We recently observed that hPTH-(1−31), shown by others to be a full AC agonist but to be incapable of activating PKC via rodent PTHRs (*27*), was as potent as hPTH-(1−34) in activating both AC and PLC via human PTHRs expressed in LLC-PK1, COS-7, or HEK 293 cells (*37*). These unexpected observations prompted us to undertake a more detailed analysis of the relative roles of the N- and C-terminal regions of hPTH-(1−34) in binding to, and activation of, the human PTHR, with a particular focus on PLC activation.

EXPERIMENTAL PROCEDURES

Cell Culture. HKRK B7 or EW5 cells (*37*), subclones of LLC-PK1 renal epithelial cells that stably express type-1 human or rat PTH/PTHrP receptors (950 000 and 320 000 receptors/cell, respectively), were maintained under 5% CO_2 in air in Dulbecco's modified essential medium containing 7% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HEK-293 and COS-7 cells were cultured similarly, except that 10% FBS was used in the medium. Cells were seeded into 24-well plates 2 days before assay at a density of (2−2.5) × 10^5 cells/well (HKRK B7 or EW 5 cells) or 6 × 10^5 cells/well (HEK-293 cells). In experiments involving HEK-293 cells, DNA transfections were performed 24 h after plating and inositol radiolabel was added 24 h thereafter (see below). COS-7 cell transfections were performed as previously described (*38*).

Radioligand Binding and Signaling Assays. Intracellular cAMP accumulation, PLC activation, and PTHR binding affinity were measured as previously reported (*4, 10*). Cyclic AMP and PLC responses generally were expressed as percentages of the maximal response to hPTH-(1−34), measured in the same experiment, after subtracting basal activities from all measurements.

Intracellular cAMP accumulation was measured in extracts of cells that were exposed to human PTH peptides in the presence of isobutylmethyxanthine (IBMX, 1 mM) at 37 °C for 15 min. After terminating the reactions by aspiration and freezing in liquid nitrogen, cell monolayers were extracted with 50 mM HCl and cAMP was measured using a radioimmunoassay kit (Dupont-New England Nuclear, Boston, MA). The basal and maximally stimulated levels of cAMP (pmol/well/15 min) in the experiments reported here were, respectively, 12 ± 4 and 207 ± 37 for HKRK B7 cells and 8 ± 6 and 270 ± 89 for EW5 cells (eight experiments each).

PLC was activated by PTH agonists in the presence of 30 mM LiCl at 37 °C for 30 min after 16 h of labeling with [^{3}H]myo-inositol (3 μCi/mL) in serum-free medium containing 0.1% bovine serum albumin. The reactions were stopped by rapid aspiration and addition of cold 5% TCA. Water-soluble radiolabeled inositol trisphosphate (IP_3) was isolated after ether extraction by ion-exchange chromatography (*10*). Basal and maximally stimulated IP3 in the experiments reported here were, respectively, 898 ± 142 and 1908 ± 251 cpm/well for HKRK B7 cells (11 experiments) and 171 ± 39 and 666 ± 243 cpm/well for EW5 cells (eight experiments). In experiments involving HEK 293 cells, cells previously transfected with human PTHR cDNA (in the pcDNA1 expression vector HKRK) using Lipofectamine (Gibco BRL) were labeled with [^{3}H]myo-inositol and assayed as described above, except that total water-soluble [^{3}H]inositol polyphosphates were collected as a single fraction from the ion-exchange columns. Basal and maximally stimulated total IPs for HEK-293 cells averaged 479 ± 62 and 5543 ± 885 cpm/well, respectively, in two experiments.

Radioligand competition binding assays were performed at 2−8 C for 6 h using [^{125}I][$Nle^{8,21}$,Tyr^{34}]rat PTH-(1−34) (100 000 cpm/well) in the presence or absence of nonradioactive hPTH-(1−34) analogues. Cell layers were washed three times before solubilization for determination of cell-associated radioactivity. Average maximal and nonspecific radioligand binding to HKRK B7 cells in the experiments reported here were 25 520 ± 1909 and 956 ± 135 cpm/well, respectively.

Peptides and Other Reagents. All reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified. All isotopes were obtained from Dupont-New England Nuclear (Boston, MA). Human PTH peptides were synthesized in

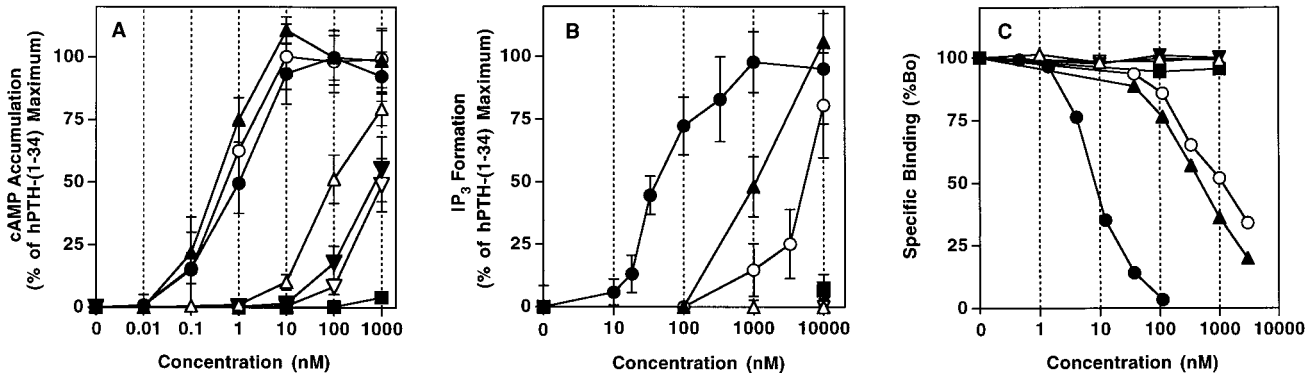

FIGURE 1: Properties of C-terminally truncated hPTH analogues in HKRK B7 cells. Intracellular cAMP accumulation (A), IP_3 formation (B), and competitive radioligand binding (C) are depicted for the indicated concentrations of: hPTH-(1−34) (●), hPTH-(1−29) (▲), hPTH-(1−28) (○), hPTH-(1−27) (△), hPTH-(1−26) (▼), hPTH-(1−25) (▽), and hPTH-(1−24) (■). Results are expressed as percentages of the maximal responses to hPTH-(1−34) (A and B) or the total specific binding of the [^{125}I][Nle^{8,21},Tyr^{34}]rat PTH-(1−34) radioligand (C) observed in the same assay after subtraction of basal activity or nonspecific binding. Each point represents the mean ± SEM of results from several (i.e., 2−4) experiments, each of which was performed in triplicate (SEMs of binding data typically were less than 3% of total binding and thus often are obscured by the symbols).

the Biopolymer Core Laboratory of the Endocrine Unit with C-terminal amidation and, when present, substitution of Tyr^{34} for the naturally occurring Phe^{34}. [Tyr^{0}]hPTH-(1−34) was purchased from Sigma Co. [^{125}I][Nle^{8,21},Tyr^{34}]rat PTH-(1−34) was iodinated and purified as previously described (*4, 10*).

Statistical Analysis. Results were expressed as the mean ± SEM for groups of separate culture wells. Significance of differences was determined by analysis of variance (ANOVA) followed by Bonferroni correction of *t*-tests for multiple comparisons against the same control group.

RESULTS

Carboxyl-Truncated PTH Analogues. We previously reported that the C-truncated peptides hPTH-(1−31) and hPTH-(1−30) fully activated both AC and PLC via the human PTHR in HKRK B7 cells and that the EC_{50}s for these responses were identical, or nearly so, to those seen for hPTH-(1−34) (*37*). To further define the C-terminal limit for retention of bioactivity, the present analysis of C-truncated hPTH peptides was begun with hPTH-(1−29) and extended to hPTH-(1−24).

As shown in Figure 1A, hPTH-(1−29) and hPTH-(1−28) activated AC via human PTHRs in HKRK B7 cells as effectively as did hPTH-(1−34), whereas further shortening (i.e., removal of Leu^{28}) strikingly diminished AC activity by approximately 100-fold (EC_{50} = 100 vs 1 nM). Further truncation, to hPTH-(1−26), produced another 10-fold reduction in potency. The responses to hPTH-(1−26) and hPTH-(1−25) were nearly identical, whereas hPTH-(1−24) was only minimally active at the highest concentration tested (1000 nM).

Profiles of the PLC activities of these peptides were strikingly different from those for AC. Thus, PLC activation by hPTH-(1−29) in these cells was dramatically reduced relative to that for hPTH-(1−34) (EC_{50} = 1000 vs 30 nM). Moreover, hPTH-(1−28) was approximately 5-fold less potent than hPTH-(1−29), whereas no PLC activation was detectable with peptides shorter than hPTH-(1−28) (Figure 1B). Similar results were obtained using HEK-293 cells acutely transfected with human PTHRs, in which the maximal PLC response to hPTH-(1−34) was 8.5-fold basal (Table 1). The progressive loss of PLC activity due to C-truncation correlated with measured losses in binding affinity in HKRK B7 cells, i.e., the apparent IC_{50}s for binding of hPTH-(1−29) and hPTH-(1−28) also were reduced 30−100 fold, compared with that of hPTH-(1−34), and no displacement of radioligand occurred with peptides shorter than hPTH-(1−28), even at concentrations as high as 1000 nM (Figure 1C).

Table 1: Activation of PLC by hPTH Peptides in Transiently Transfected HEK-293 Cells[a]

human PTH peptide	PLC activity (% basal)
control	100 ± 11
hPTH-(1−34)	854 ± 188[b]
hPTH-(1−28)	305 ± 20[b]
hPTH-(1−27)	95 ± 23
hPTH-(2−34)	88 ± 23
desamino[Ala^{1}]hPTH-(1−34)	106 ± 20
desamino[Gly^{1}]hPTH-(1−34)	108 ± 12

[a] Total inositol polyphosphates produced in response to 1000 nM of the peptides shown were measured in HEK-293 cells that transiently expressed human PTHRs. Results are expressed as means ± SEMs of the % of basal activity for triplicate determinations. The basal level of total IPs in controls was 578 ± 91 cpm/well/30 min. [b] Significantly different from controls ($p < 0.05$).

To determine if the progressive loss of PLC activity associated with stepwise C-terminal truncation of hPTH-(1−34) resulted from the parallel loss in binding affinity or, instead, from coincidental deletion of a critical PLC activation domain, we introduced into hPTH-(1−28) a modification—substitution of Arg for Glu at position 19—that previously was reported to enhance binding of hPTH-(1−34) to rodent PTHRs (*39*). The resulting peptide, [Arg^{19}]-hPTH-(1−28), exhibited both enhanced binding (IC_{50} = 100 vs 1000 nM) and increased PLC activity (EC_{50} = 300 vs 6000 nM), relative to hPTH-(1−28) (see filled symbols, Figure 2). Importantly, the [Arg^{19}]hPTH-(1−28) analogue maximally activated PLC, despite absence of the hPTH-(29−32) sequence reported to be essential for PKC activation (*12, 27*). Adenylyl cyclase activity was unaffected by the Arg^{19} substitution (Figure 2A). As these results suggested that amino acids C-terminal to position 28 of hPTH, while important for optimal ligand binding, are not required for maximal PLC activation via the human PTHR, we then

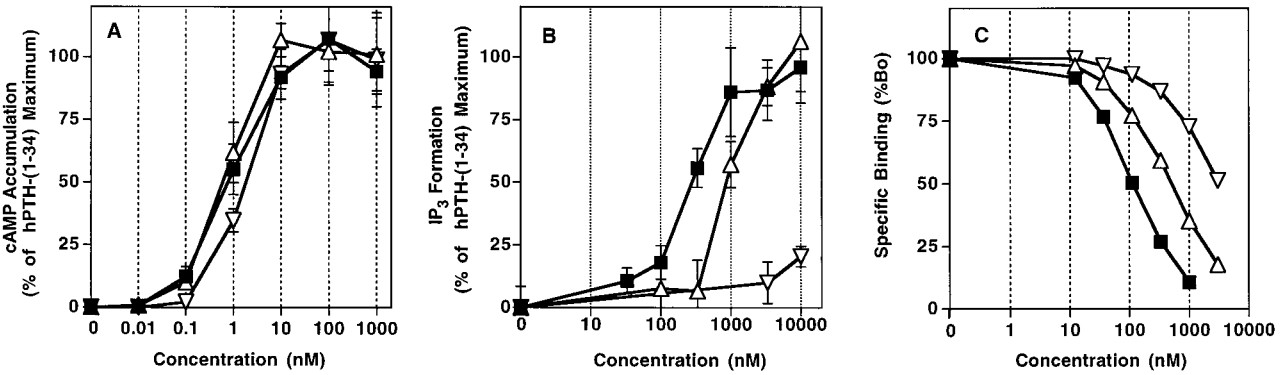

FIGURE 2: Effects of mutations at positions 1 or 19 on properties of hPTH-(1−28) in HKRK B7 cells. Intracellular cAMP accumulation (A), IP_3 formation (B) and competitive radioligand binding (C) are depicted for [Arg^{19}]hPTH-(1−28) (■), [Ala^1]hPTH-(1−28) (△), and [Gly^1]hPTH-(1−28) (▽) added at the concentrations indicated. Results are expressed as percentages of the maximal response to hPTH-(1−34) measured in the same experiment, as described in Figure 1.

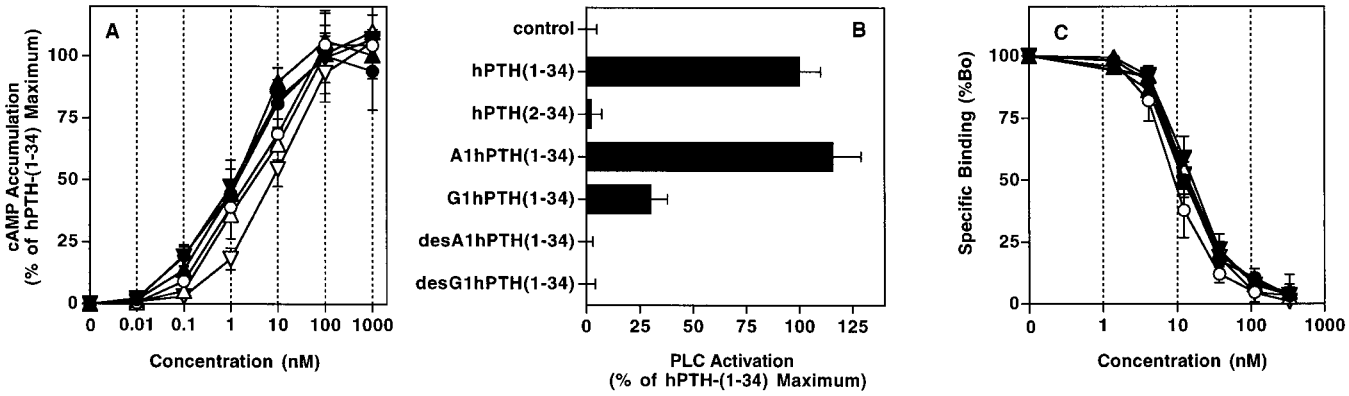

FIGURE 3: Properties of N-terminally modified hPTH-(1−34) analogues in HKRK B7 cells. (A) Intracellular cAMP accumulation in response to the indicated concentrations of: hPTH-(1−34) (●), hPTH-(2−34) (○), [Ala^1]hPTH-(1−34) (▲), [Gly^1]hPTH-(1−34) (▼), desamino-[Ala^1]hPTH-(1−34) (△), and desamino-[Gly^1]hPTH-(1−34) (▽); (B) Formation of IP_3 in response to 1000 nM of the indicated peptides; (C) Competitive radioligand binding of the same peptides denoted in panel A. Results are expressed as percentages of the maximal response to hPTH-(1−34) measured in the same experiment, as described in Figure 1. When the N-truncated peptides were tested at 10 000 nM (one experiment, not shown), hPTH-(2−34) increased PLC by 14 ± 2% over controls, whereas the two desamino analogues induced less than 10% activation.

focused upon the role of the N-terminus of hPTH in PLC signaling.

N-Terminally Modified hPTH Analogues. Human PTH-(3−34) had been characterized previously as a PLC/PKC-selective peptide via rodent PTH receptors (*3, 8, 27*). We have observed, however, that this peptide, at concentrations as high as 1000 nM, did not activate PLC in HKRK B7 cells or in COS-7 or HEK-293 cells that expressed rat or human PTHRs (*21*, and unpublished observations). These results indicated that the first two amino acids at the N-terminus of hPTH-(1−34) (Ser^1-Val^2) must be important for PLC as well as AC activation via the human PTHR.

To refine this analysis, we first studied the properties of hPTH-(2−34). As shown in Figure 3, panels A and B, hPTH-(2−34) did not elicit PLC activity when tested at a concentration of 1000 nM in HKRK B7 cells, even though its potency and efficacy for activation of AC was comparable to that of hPTH-(1−34). Only minimal PLC activation occurred in one experiment at a 10-fold higher concentration of this peptide (see legend, Figure 3). Similar results were obtained using COS-7 cells expressing abundant human PTHRs (data not shown). The inability of hPTH-(2−34) to activate PLC via human PTHRs was confirmed in a fully homologous system by using HEK-293 human embryonic kidney cells that were transiently transfected with human PTHR cDNA (Table 1).

We next addressed the role of the N-terminal Ser^1 residue and of its α-amino group in binding to and activation of the human PTHR. Because earlier work with isolated rat renal membranes had shown that substitution of Ala^1 for Ser^1 in hPTH-(1−34) increased its AC activity, whereas a Gly^1 substitution impaired AC activation by bPTH-(1−34) (*29*), these position-1 modifications were introduced into hPTH-(1−34), with the results shown in Figure 3. The [Ala^1]hPTH-(1−34) analogue was indistinguishable from hPTH-(1−34) with respect to binding as well as activation of AC or PLC, whereas the Gly^1 -substituted peptide showed diminished PLC activity (25% of the hPTH-(1−34) maximum at 1000 nM) despite unchanged AC activity and binding IC_{50}.

Deletion of the α-amino groups of these Ala^1- and Gly^1-substituted hPTH-(1−34) peptides {i.e., desamino[Ala^1]-hPTH-(1−34) and desamino[Gly^1]hPTH-(1−34), respectively} resulted in complete loss of detectable PLC activity in HKRK B7 cells, although there was no change in apparent binding affinity and no more than a 2−5-fold reduction in AC potency (Figure 3). These results were confirmed using COS-7 cells expressing rat or human PTHRs (not shown) and with HEK-293 human kidney cells transiently transfected

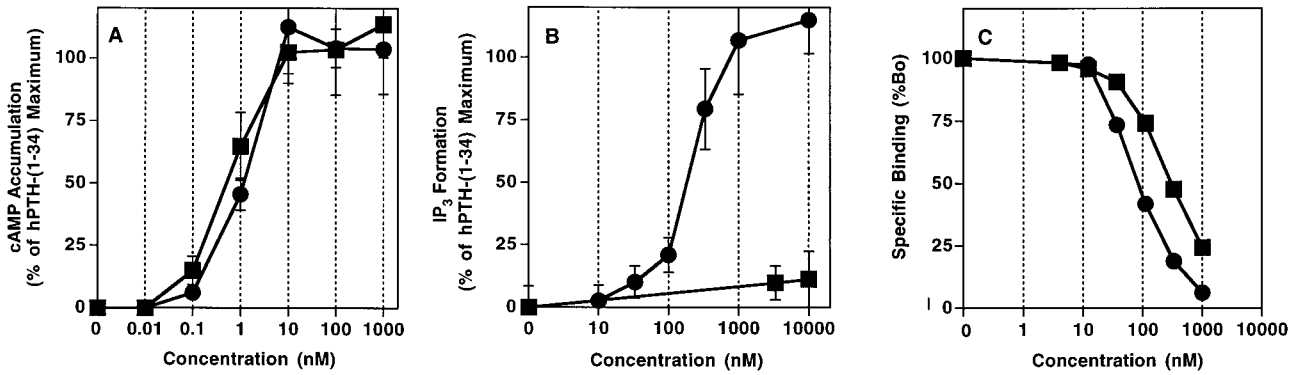

FIGURE 4: Effects of mutations at positions 1 and 19 on properties of hPTH-(1−28) in HKRK B7 cells. Intracellular cAMP accumulation (A), IP_3 formation (B), and competitive radioligand binding (C) are depicted for [Ala[1],Arg[19]]hPTH-(1−28) (●) and [Gly[1],Arg[19]]hPTH-(1−28) (■) at the indicated concentrations. Results are expressed as percentages of the maximal response to hPTH-(1−34) measured in the same experiment, as described in Figure 1.

with human PTHR cDNA (Table 1). Removal of the α-amino group of hPTHrP(1−36) also eliminated PLC activation by that ligand in HKRK B7 cells [i.e., hPTHrP(1−36) = 178 ± 12; desamino-hPTHrP(1−36) = 101 ± 6% of basal, respectively, at 1000 nM peptide]. Displacement of the N-terminal α-amino group by interposition of a tyrosine residue at the N-terminus of hPTH-(1−34) [i.e., [Tyr[0]]hPTH-(1−34)] also abolished PLC activity (105 ± 5% of basal at 1000 nM peptide). Collectively, these findings pointed to a critical role for the N-terminal residue of hPTH-(1−34) and of its free α-amino group in particular, in the activation of PLC by the human PTHR. As the impact of each position-1 change was both relatively selective for PLC (vs AC) in these cells and independent of changes in binding affinity, a strategy was suggested for potentially dissociating PLC from AC activation via the type 1 PTHR.

Modifications of hPTH-(1−28). As shown earlier (Figure 1), hPTH-(1−28) was the shortest unsubstituted fragment of hPTH-(1−34) that, at the highest concentration tested (10 uM), still could activate both AC and PLC via the human PTHR in HKRK B7 cells. When Ala[1] was substituted for Ser[1] in the hPTH-(1−28) sequence, relative binding affinity (IC_{50}) and the EC_{50} for PLC activation both were partially improved, although not to the extent observed previously with [Arg[19]]hPTH-(1−28) (see Figure 2, panels B and C). Activation of AC, already equivalent to that of hPTH-(1−34), was not further enhanced. In contrast, the Gly[1] substitution [i.e., [Gly[1]]hPTH-(1−28)] selectively eliminated PLC signaling, apart from a weak response at 10 μM and also modestly impaired binding affinity (Figure 2, panels B and C).

When the binding-enhancing Arg[19] modification was superimposed upon the [Ala[1]]hPTH-(1−28) and [Gly[1]]hPTH-(1−28) analogues, the binding IC_{50}s of these peptides for the human PTHR were shifted by 5−10-fold (Figure 4C, compare with Figure 2C). In the case of [Ala[1],Arg[19]]hPTH-(1−28), the EC_{50} for PLC activation also was improved 5-fold [i.e., from 1000 nM (Figure 2B) to 200 nM (Figure 4B)] over that of [Ala[1]]hPTH-(1−28). Interestingly, however, the Ala[1],Arg[19] double substitution could not restore PLC activity to hPTH-(1−27), although it did improve the EC_{50} for AC activation by 10-fold (Table 2, Figure 1). In contrast to [Ala[1],Arg[19]]hPTH-(1−28), there was no increase in PLC activity following introduction of the Arg[19] substitution into [Gly[1]]hPTH-(1−28) despite a comparable 5-fold improvement in binding affinity (Figure 4). Thus, the AC activation curves for both [Ala[1],Arg[19]]hPTH-(1−28) and [Gly[1],Arg[19]]-hPTH-(1−28) were indistinguishable from that of hPTH-(1−34) (Figure 4A), whereas the PLC response profiles diverged markedly (Figure 4B), demonstrating that [Gly[1],-Arg[19]]hPTH-(1−28), unlike [Ala[1],Arg[19]]hPTH-(1−28), was strikingly and selectively impaired with respect to activation of this signaling pathway.

Table 2: Activities of [Ala1,Arg19]hPTH-(1−27) in HKRK B7 Cells[a]

peptide concentration (nM)	response (% maximum)	
	hPTH-(1−34)	[Ala[1],Arg[19]]hPTH-(1−27)
	Cyclic AMP Accumulation	
0.01	1.7 ± 0.7	−0.2 ± 0.1
0.1	14 ± 1.4	0.6 ± 0.3
1	45 ± 7.8	7 ± 0.6
10	93 ± 8.1	50 ± 5.1
100	96 ± 9.5	92 ± 15
1000	100 ± 12	99 ± 8.8
	IP_3 Formation	
1000	87 ± 2.8	1.5 ± 4.8
10000	100 ± 13	6.9 ± 10

[a] Signaling responses were expressed as % of the maximal response to hPTH-(1−34) observed in the same assay. Values are the mean ± SEM of 4 (cAMP accumulation) or 6 (IP_3 formation) measurements from two separate experiments performed in duplicate and triplicate, respectively.

The likelihood that the [Gly[1],Arg[19]]hPTH-(1−28) peptide might be useful, especially in rodent models, as a signal-selective analogue to dissociate AC from PLC signaling via the PTHR was examined further using the EW5 subclone of LLC-PK1 cells, which stably expresses rat type-1 PTHRs (*10*). As shown in Table 3, the signaling and binding properties of the various substituted hPTH-(1−28) analogues in EW5 cells were similar, though not identical, to those described above in HKRK B7 cells. Differences noted for the hPTH-(1−28) peptides between the rat and human receptors included (1) signaling of AC and PLC by hPTH-(1−28) via the rat PTHR was more severely impaired, both relative to hPTH-(1−34) and in absolute terms, than via the human PTHR, despite comparable binding affinity (compare Table 3 and Figure 1); (2) the Gly[1] substitution slightly enhanced (by 2-fold) binding affinity of hPTH-(1−28) peptides for the rat PTHR, whereas it impaired binding to the human PTHR by about 3-fold (compare Table 3 with Figures 2 and 4); and (3) the binding IC_{50} of [Gly[1],Arg[19]]-

Table 3: Properties of hPTH-(1−28) Analogues via Type 1 Rat PTHRs[a]

peptide	binding IC_{50} (nM)	AC EC_{50} (nM)	PLC EC_{50} (nM)
hPTH-(1−34)	7 ± 2	0.8 ± 0.4	180 ± 20
hPTH-(1−28)	820 ± 110	18 ± 8.7	>10000[b]
[Arg[19]]hPTH-(1−28)	67 ± 11	1.4 ± 1.3	830 ± 240
[Ala[1]]hPTH-(1−28)	190 ± 42	3.3 ± 0.6	2950 ± 212
[Gly[1]]hPTH-(1−28)	540 ± 120	42 ± 16	>10000[b]
[Ala[1],Arg[19]]hPTH-(1−28)	28 ± 3	0.5 ± 0.2	265 ± 64
[Gly[1],Arg[19]]hPTH-(1−28)	47 ± 5	4.3 ± 1.7	>10000[b]

[a] Values shown are mean IC_{50} or EC50s (nM; ±SD) as determined from two to four experiments ($N \geq 6$) with each peptide using EW5 LLC−PK1 cells that stably express 320 000 rat PTHRs/cell. [b] Highest concentration tested = 10 000 nM. No significant PLC activation was observed with [Gly[1,] Arg[19]]hPTH-(1−28) at 10 000 nM (9.8 + 7.2% of maximal; $N = 11$).

hPTH-(1−28) for the rat PTHR was impaired only about 6-fold relative to hPTH-(1−34), whereas binding of this analogue to the human PTHR was reduced almost 40-fold. Despite only modestly reduced (6-fold) binding affinity and AC potency, relative to hPTH-(1−34), no activation of PLC by the [Gly[1],Arg[19]]hPTH-(1−28) analogue could be detected at concentrations as high as 10 000 nM. Thus, this analogue exhibited strikingly altered selectivity for AC over PLC, compared with hPTH-(1−34), via the rat PTHR. Although the potency of [Gly[1],Arg[19]]hPTH-(1−28) for activation of AC via the human PTHR was similar to that of hPTH-(1−34) in HKRK−B7 cells, it was reduced up to 10−20-fold, concordant with its reduced binding affinity, in other cells (LLC-PK1 or SaOS-2 osteosarcoma) that expressed many fewer human PTHRs (i.e., 10000−100000 vs 950000/cell) (data not shown). Overall, the results indicate that the [Gly[1],-Arg[19]]hPTH-(1−28) peptide is highly signal selective for AC vs PLC via both the rat and human PTHRs and that it could be especially useful, due to its well-preserved binding affinity for the rat PTHR, for studies with rodent models in vivo.

DISCUSSION

These results support several major conclusions regarding the interaction of hPTH-(1−34) and the type 1 PTHR. First, an intact N-terminus of the hPTH-(1−34) ligand clearly is indispensable for effective activation of PLC via the PTHR. The C-terminus of hPTH-(1−34), especially the sequence hPTH-(28−30), promotes effective PLC activation, mainly by contributing to ligand binding, but it is not required for maximal PLC activation if binding can be maintained by binding-enhancing modifications (such as the Arg[19] substitution). Second, although the N-terminus of hPTH is critical for activation of both AC and PLC via the type-1 PTHR, the necessary structural determinants within this region are different for the two responses. Specifically, PLC activation requires the N-terminal α-amino group and is more sensitive to the structure of the N-terminal amino acid than the AC response, which can occur even in the absence of an amino acid at position 1. Third, because of this differential sensitivity to the configuration of the N-terminus, it is possible to disproportionately modify potency for activation of these two effectors by altering the structure of the ligand, as shown for the [Gly[1],Arg[19]]hPTH-(1−28) analogue. We previously had accomplished such signaling selectivity through mutations in the PTHR [by changing the EKKY sequence in intracellular loop 2 to DSEL (*40*)], but it now is clear that a highly signal-selective ligand, active via the wild-type receptor, also can be designed.

These findings require reexamination of some key concepts concerning the structural determinants of PTH signaling. In work conducted previously with rodent and opossum cells that express endogenous PTHRs, certain N-truncated PTH analogues, such as PTH-(3−34), lacked AC activity but nevertheless potently activated PKC and, in some systems (*6*, *8*, *16*) but not others (*32*, *33*), Ca_i^{2+} transients. Detailed analysis of the PKC-activating properties of N- and C-truncated hPTH-(1−34) analogues had led to the conclusion that the sequence hPTH-(29−32) constitutes a critical "PKC activation domain" (*12*, *27*, *34*).

Our findings with C-truncated analogues, including hPTH-(1−28) and especially [Ala[1],Arg[19]]hPTH-(1−28), clearly demonstrate, however, that the hPTH-(29−32) region is not required for full activation of PLC, which is thought to be a major pathway for PKC activation via the type-1 PTHR. These results were not confined to a single cell line but were obtained using HEK-293 cells expressing acutely transfected human PTHRs as well as LLC-PK1 cells that stably expressed either rat or human PTHRs. We did confirm that the hPTH-(29−32) region is important for high-affinity binding of the hPTH-(1−34) ligand, as reported previously for bPTH-(1-34) in rat renal membranes (*28*). Further, consistent with our previous analyses of the effects of altered levels of PTHR expression (*10*, *21*), we now provide further evidence, using the independent approach of altering the C-terminal extent of the ligand, that PLC is less effectively activated by the occupied PTHR than AC. Thus, the ratio of potencies for activation of AC vs PLC by each ligand increased progressively with stepwise C-terminal truncation. In fact, we ultimately could document AC activation by peptides such as hPTH-(1−27) and hPTH-(1−25) that could neither stimulate PLC nor manifest sufficient relative binding affinity to the PTHR to be detectable by radioligand binding analysis. A similar dissociation of binding affinity from AC potency was reported recently for another PTHR ligand (*41*). These differences undoubtedly were accentuated in HKRK B7 cells that possess a high degree of receptor reserve.

A consistent view of the role of the N-terminus of PTH-(1−34) in activating PLC or Ca_i^{2+} signaling via recombinant PTHRs has not emerged from previous studies (*14*, *15*, *19*, *36*). Because N-terminally truncated PTH analogues had been shown to activate PKC and Ca_i^{2+} in several other systems (*3*, *6*, *8*, *9*, *11*, *27*, *42*−*44*), our finding that a free α-amino group at position-1 of hPTH-(1−34) is required for PLC activation via the type-1 PTHR was unexpected. In fact, our data indicate that PLC activation is even more sensitive to such subtle N-terminal modifications than AC. Moreover, in contrast to the effects of C-terminal truncation of hPTH-(1−34), this selective reduction in PLC potency is not due to impaired binding of the N-modified analogues. This is evident from the similar binding but totally discrepant PLC activation of several N-modified hPTH(1−34) analogues (Figure 3) and of the Ala[1]- vs Gly[1]-substituted versions of [Arg[19]]hPTH(1−28) (Figure 4). These findings indicate that a true "activation domain" for PLC signaling must reside at the extreme N-terminus of hPTH-(1−34). This conclusion contrasts with previous reports of PLC activation by the

N-truncated bovine peptides bPTH-(2−34) and bPTH-(3−34) in rat UMR 106-01 osteosarcoma cells (*8*). UMR cells may express alternate species of PLC-coupled PTH receptors, however (*45, 46*), a possibility that cannot be excluded in the presence of endogenous type-1 PTHRs. Alternatively, this discrepancy could be due to species differences (in cells and ligand) or to expression of a different array of G proteins in UMR osteosarcoma cells than in the LLC-PK1, COS-7, or HEK-293 kidney cells studied here.

It should be noted that N-truncated analogues, including bPTH-(2−34), hPTH-(2−38), and desamino-PTH-(1−34), previously were shown to be only weakly active in stimulating AC in vitro (*8, 9, 29, 44*), which contrasts with our findings that hPTH-(2−34), desamino-[Ala^1]hPTH-(1−34), and desamino-[Gly^1]hPTH-(1−34) were equipotent with hPTH-(1−34). This apparent disparity likely is explained by the much higher degree of receptor reserve in HKRK B7 cells than in the osteosarcoma cells or partially purified membranes used in previous studies. We employed HKRK B7 cells to facilitate analysis of the structural basis of PLC signaling, reliable measurement of which requires a high level of receptor expression (*10, 23*). When these N-truncated peptides were tested with other LLC-PK1 subclones that expressed fewer human PTHRs (i.e., 100000−300000/cell), their AC responses were indeed reduced by as much as 10-fold in potency, relative to hPTH-(1−34) (data not shown). Such a shift in relative agonist potency on the basis of increased receptor number alone is not predicted by classical receptor theory; although, similar results have been observed with other G protein-coupled receptors and have been attributed to nonequilibrium conditions, limited receptor/effector diffusion in the two-dimensional membrane environment, compartmentalization of receptors or G proteins, or complex secondary effects due to agonist differences in relative efficacy for activation of multiple G proteins (*47−49*). It is worth noting, however, that earlier studies of similar N-truncated PTH analogues in vivo had demonstrated much greater biopotency than predicted from measures of their AC activity in vitro (*50, 51*). Moreover, the actual number of PTHRs expressed by relevant target cells in vivo remains uncertain but could be within the range studied here (*24, 25*).

It is important to emphasize that our results are not inconsistent with previous evidence that the C-terminus of hPTH-(1−34) activates PKC via the type-1 PTHR (*3, 11, 12, 16, 27*) or that Ca_i^{2+} transients may occur in the absence of an intact N-terminus (*6, 8, 16*). Protein kinase C may be activated by mechanisms other than PLC, including phospholipase D (PLD) and phospholipase A_2 (PLA_2) (*52*). Indeed, PTH activates PLA_2 and PLD in some cells (*53−55*), and it also may stimulate both Ca_i^{2+} transients (*15, 19*) and PKC (*56*) without increasing PLC. If the C-terminal region of PTH-(1−34) is involved in such alternate pathways of PKC or Ca_i^{2+} activation, our results lead to the conclusion that at least three activation domains reside within the hPTH-(1−34) molecule—two at the N-terminus that mediate activation of AC and PLC, respectively, and one near the C-terminus that activates PKC via one or more PLC-independent mechanisms.

This is the first demonstration that peptides shorter than PTH-(1−28) can fully activate AC via the type-1 PTHR. Previous studies of isolated renal membranes or ROS 17/2 osteosarcoma cells had detected little or no activity with PTH-(1−27) or shorter C-truncated analogues (*28, 50, 57*). As noted above for the N-truncated peptides, our ability to detect AC activation by such C-truncated hPTH peptides likely reflects the high level of human PTHR reserve in the cells we used. Of course, until physiologic levels of PTHR expression have been defined in relevant target cells in vivo, estimates of the AC activity of PTH analogues or mimetic compounds obtained using these highly sensitive in vitro systems must be interpreted cautiously. At the same time, such systems appear to offer important advantages for identifying weak human PTHR agonists and for defining the elemental structural features of ligands involved in binding and multiple parallel signaling by this receptor.

Finally, our studies of modified hPTH-(1−28) analogues have identified a novel hPTH analogue, [Gly^1,Arg^{19}]hPTH-(1−28), that exhibits marked selectivity, relative to hPTH-(1−34), for activation of AC (vs PLC) via the human PTHR. In HKRK B7 cells that express abundant human type 1 PTHRs, activation of AC by this analogue was equivalent to that by hPTH(1−34), whereas PLC potency was reduced approximately 100-fold. Although, like hPTH-(2−34) and the desamino analogues, the absolute AC potency of [Gly^1,Arg^{19}]hPTH-(1−28) is reduced in cells that express many fewer human PTHRs/cell, this peptide nevertheless retains sufficient binding and AC activity to prove potentially useful as a signal-selective PTHR agonist for studies of the roles of AC vs PLC in mediating specific cellular actions of PTH. This is especially true for rodent systems, as [Gly^1,Arg^{19}]-hPTH-(1−28) binds to the rat PTHR nearly as well as does hPTH-(1−34) and yet still lacks detectable PLC activity. Further analysis of the properties of this analogue in vitro and in vivo could help clarify the role of PTHR-dependent PLC activation in PTH action(s) and provide further direction for the development of other signal-selective hPTH analogues.

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