Modifications of Position 12 in Parathyroid Hormone and Parathyroid Hormone Related Protein: Toward the Design of Highly Potent Antagonists

Michael Chorev, Mark E. Goldman, Roberta L. McKee, Eliahu Roubini, Jay J. Levy, C. Thomas Gay, Jane E. Reagan, John E. Fisher, Lynn H. Caporale, Ellis E. Golub, Michael P. Caulfield, Ruth F. Nutt, and Michael Rosenblatt*

Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

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ABSTRACT: Truncated N-terminal fragments of parathyroid hormone (PTH), [Tyr^{34}]bovine PTH(7–34)NH_2, and parathyroid hormone related protein (PTHrP), PTHrP(7–34)NH_2, inhibit [Nle^{8,18},[^{125}I]iodo-Tyr^{34}]-bPTH(1–34)NH_2 binding and PTH-stimulated adenylate cyclase in bone and kidney assays. However, the receptor interactions of these peptides are 2–3 orders of magnitude weaker than those of their agonist counterparts. To produce an antagonist with increased receptor-binding affinity but lacking agonist-like properties, structure–function studies were undertaken. Glycine at position 12 (present in all homologues of PTH and in PTHrP), which is predicted in both hormones to participate in a β-turn, was examined by substituting conformational reporters, such as D- or L-Ala, Pro, and α-aminoisobutyric acid (Aib), in both agonist and antagonist analogues. Except for N-substituted amino acids, which substantially diminished potency, substitutions were well tolerated, indicating that this site can accept a wide latitude of modifications. To augment receptor avidity, hydrophobic residues compatible with helical secondary structure were introduced. Incorporation of the nonnatural amino acids D-Trp, D-α-naphthylalanine (D-α-Nal), or D-β-Nal into either [Tyr^{34}]bPTH(7–34)NH_2 or [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH_2 resulted in antagonists that were about 10-fold more active than their respective 7–34 parent compound. Similarly, [D-Trp^{12}]PTHrP(7–34)NH_2 was 6 times more potent than the unsubstituted peptide but retained partial agonistic properties, although markedly reduced, similar to PTHrP(7–34)NH_2. The antagonistic potentiating effect was configurationally specific. This study provides the basis for a rational approach toward the design of more potent antagonists of both PTH and PTHrP based on the introduction of hydrophobic residues (to increase receptor avidity) into sites within the antagonist sequence which have been established to be tolerant of structural manipulation.

Parathyroid hormone (PTH), a linear polypeptide of 84 amino acids, serves a critical role in calcium homeostasis. PTH receptors that stimulate adenylate cyclase and perhaps other intracellular second messengers are present in the hormone's major target tissues: kidney and bone. Recently, a new tumor-secreted hormone associated with the clinical syndrome of humoral hypercalcemia of malignancy, namely, parathyroid hormone related protein (PTHrP) (Strewler et al., 1983; Stewart et al., 1983; Rodan et al., 1983), has been structurally elucidated (Moseley et al., 1987; Stewart et al., 1987; Strewler et al., 1987; Suva et al., 1987). PTHrP and PTH are homologous in their N-terminal domains (Figure 1).

Evidence collected both in vitro and in vivo from several laboratories indicates that expression of PTHrP bioactivity results from interaction of the hormone with what has been conventionally regarded as PTH receptors. The N-terminal fragments (34 amino acids in length) of both PTHrP and PTH display similar biological profiles (Horiuchi et al., 1987; Kemp et al., 1987). Both peptides bind to and desensitize PTH receptors (Juppner et al., 1988; Fukayama et al., 1988; Rabbani et al., 1988). This similarity in bioactivity occurs despite considerable structural divergence outside of the N-terminal 13 amino acid domains.

Our laboratory has had a long-standing interest in the design of PTH antagonists. The potential research applications and clinical utility of such antagonists has been reviewed previously (Rosenblatt, 1986). The discovery of PTHrP provides new impetus for the design of potent and effective antagonists of both PTH and PTHrP based on the contribution of PTHrP to the pathogenesis of hypercalcemia of malignancy and of the new structural possibilities revealed by the PTHrP sequence.

Previous studies have shown that deletion of two amino acids from the amino terminus of [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH_2, an analogue of PTH which retains full potency in most bioassays (Rosenblatt et al., 1976), yielded the most potent in vitro PTH antagonist yet described, namely, [Nle^{8,18},Tyr^{34}]bPTH(3–34)NH_2 (Rosenblatt et al., 1977). This analogue binds to bovine renal cortical membranes with K_b = 2.9 nM and inhibits PTH-stimulated adenylate cyclase with K_i = 32 nM (Goldman et al., 1988a). However, in vivo studies revealed that the analogue has weak partial agonist properties (Segre et al., 1979; Horiuchi et al., 1983a). Truncation of four more residues from the N-terminus yielded [Tyr^{34}]bPTH(7–34)NH_2. This analogue is a pure antagonist, devoid of agonist activity in vivo (Horiuchi et al., 1983b; Doppelt et al., 1986). However, its in vitro antagonist potency is about 50-fold less than that of the 3–34 analogue (Mahaffey et al., 1979; Rosenblatt et al., 1980).

The corresponding truncated N-terminal fragment of PTHrP, PTHrP(7–34)NH_2, was synthesized and found to bind to both renal and bone PTH receptors (McKee et al., 1987). It has been demonstrated to be a potent antagonist of PTHrP(1–34)NH_2 by use of the bone-derived ROS17/2.8 cells (K_b = 100 nM and K_i = 470 nM) and bovine renal membranes (K_b = 242 nM and K_i = 410 nM). However, similar to [Nle^{8,18},Tyr^{34}]bPTH(3–34)NH_2 and in contrast to [Tyr^{34}]-

* Address correspondence to this author at Merck Sharp & Dohme Research Laboratories, W26-207, West Point, PA 19486.

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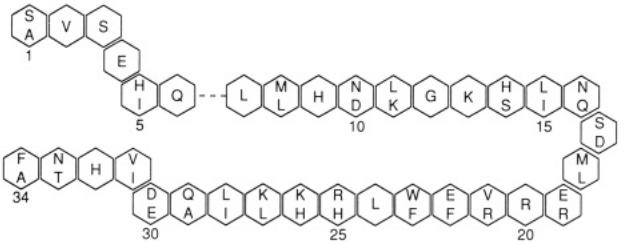

FIGURE 1: Comparison of the N-terminal sequence 1–34 of human PTH and human PTHrP. Common residues for both sequences are given by single-letter notation in the middle of the hexagon. Sites that differ in their amino acids are marked by two single letters. The letter in the upper corner of the hexagon corresponds to PTH and the letter in the lower corner to PTHrP. Deletion of six amino acids from the N-terminus eliminates a considerable portion of homology shared by the hormones.

bPTH(7–34)NH_2, PTHrP(7–34)NH_2 is a weak partial agonist (8 mM) (McKee et al., 1988).

We undertook an investigation designed to probe conformational features of PTH and PTHrP which contribute to receptor interaction. We recently compared the predicted (Chou & Fasman, 1978) secondary structure of bPTH(1–34)NH_2 and PTHrP(1–34)NH_2 utilizing a modified program (Ross & Golub, 1988). In addition to β-sheet and major α-helical regions, both peptides are predicted to contain a β-turn at positions 12–15 in PTH and 9–12 in PTHrP (see Figure 2). Experimentally, by use of circular dichroism (in water) (Brewer et al., 1974; Aloj & Edelhoch, 1972) or nuclear magnetic resonance techniques (Bundi et al., 1978), both PTH(1–84) and PTH(1–34) display mostly random structure. However, predominant helical structure is observed in nonaqueous amphiphilic environments, such as methanol (Cohn & MacGregor, 1981), and in aqueous solutions of SDS (Shah et al., 1987) or phospholipid (Epand et al., 1985).

A glycine is present in position 12 of both PTH and PTHrP. This glycine is conserved in all the known PTH sequences derived from different species. It is one of the few positions of identity for PTH and PTHrP. In addition, glycine's lack of a side chain provides conformational flexibility. Therefore, we examined the biological consequences of structural modifications at position 12. Single-residue replacements were introduced, and the biological consequences were assessed in vitro. A range of substitutions were evaluated in order to determine whether or not position 12 is important with respect to its contribution to a preferred "bioactive conformation" of the hormone or to essential interactions with receptors.

One design approach for antagonists is to identify "tolerant" positions and then introduce structural modifications that contribute additional favorable interactions with receptors that are not present in native-sequence agonists. These interactions, introduced within the framework of an antagonist sequence, might increase receptor avidity without activating receptors (transducing the transmembrane signaling apparatus).

In a recent paper (Goldman et al., 1988b), we reported the substitution of D-tryptophan for glycine at position 12 in the antagonist analogue [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH_2. Inhibitory potency versus PTH-stimulated adenylate cyclase activity and binding of radioiodinated PTH agonist to PTH receptors were enhanced 12–27-fold in renal- and bone-derived systems. These findings provided impetus for more extensive and detailed examination of structure–activity relations at position 12.

In this paper, we document the identification of position 12 as a "tolerant" site and the introduction of substitutions that generate novel, highly potent PTH and PTHrP antagonists.

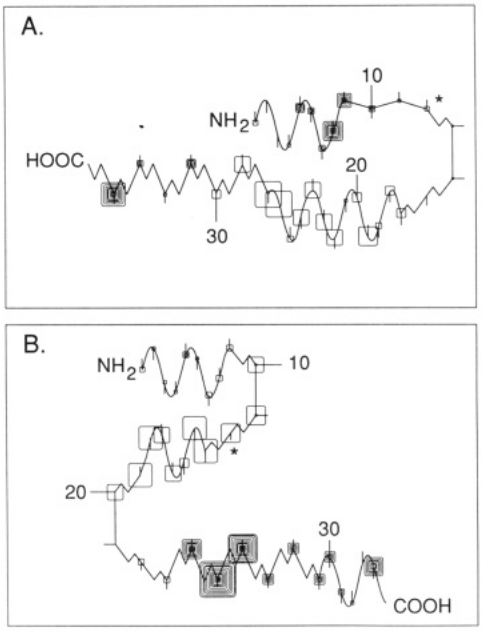

FIGURE 2: Schematic representation of secondary structure of the N-terminal 1–34 sequences of human PTH (A) and human PTHrP (B) predicted by Chou and Fasman (1978) analysis. The different structural elements are depicted as follows: sine wave, α-helix; zigzag, β-sheet; reverse direction, β-turn. Hydrophobic and hydrophilic residues are indicated by open and closed circles, respectively. The diameter of the circle is proportional to the magnitude of the hydrophobicity calculated by the method of Kyte and Doolittle (1982).

Materials and Methods

Materials. *p*-Methylbenzhydrylamine resin hydrochloride (1% cross-linked, 0.57 mM nitrogen/g, 100–200 mesh) was obtained from U.S. Biochemicals Inc. (Cleveland, OH). Ultrapure-grade [Tyr^{34}]hPTH(1–34)NH_2, [Tyr^{34}]bPTH(7–34)NH_2, [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH_2, *N*-Boc-L-Asp-(OcHex)-OH, *N*-Boc-N^{π}-Bom-L-His-OH, *N*-Boc-L-Nle-OH, *N*-Boc-Sar-OH, *N*-Boc-N^{in}-For-D-Trp-OH, *N*-Boc-D-Ala-OH, and *N*-Boc-β-Ala-OH were obtained from Bachem Inc. (Torrence, CA). *N*-Boc-α-aminoisobutyric acid (Aib) was obtained from Peninsula Laboratories Inc. (Belmont, CA). *N*-Boc-D-α-Nal-OH and *N*-Boc-D-β-Nal-OH were purchased from Omni Biochem (National City, CA). The rest of the *N*-Boc-protected amino acid derivatives, *N*,*N*′-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, diisopropylethylamine, trifluoroacetic acid, *N*,*N*-dimethylformamide, and dichloromethane were purchased from Applied Biosystems Inc. (Foster City, CA). Hydrogen fluoride was purchased from Matheson (Secaucus, NJ). *p*-Thiocresol, *p*-cresol, and methyl sulfide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bovine kidneys were the gift of Baums Meat Packing Inc. (Hatfield, PA). Bovine serum albumin, Tris-HCl, phosphocreatine, creatine phosphokinase, GTP, isobutymethylxanthine, and Mg-ATP were obtained from Sigma (St. Louis, MO).

PTH Receptor Binding and Adenylate Cyclase Assays. Kidney-based assays were performed with bovine renal cortical membranes following the procedures of Goldman et al. (1988a).

Bone-based assays were performed with ROS17/2.8 cells following the procedures described by McKee et al. (1988).

Constants for binding (K_b) and adenylate cyclase (stimulation, K_m; inhibition, K_i) were calculated according to Cheng and Prusoff (1973).

Peptide Synthesis, Purification, and Analytical Procedures. The analogues were synthesized by a modification of the solid-phase peptide synthetic methodology (Merrifield, 1969)

Table I: Amino Acid Analysis of the Agonists (Peptides **1–4**) and Antagonists (Peptides **5–15**)

amino acid residue	analogue																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Asx	4.04[a]	4.03	4.11	4.10	4.03	4.04	4.16	2.99	3.14	3.03	2.96	3.01	3.03	2.99	3.03	2.97	1.98
	4[b]	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	2
Ser	2.99	2.95	2.98	2.94	0.96	1.08	1.15	2.04	2.03	2.28	1.91	1.99	2.09	2.13	2.10	3.03	1.04
	3	3	3	3	1	1	2	2	2	2	2	2	2	2	2	3	1
Glx	5.08	5.05	5.11	5.10	3.08	3.12	3.14	3.09	3.10	3.02	3.05	3.15	3.14	3.11	3.08	3.03	2.10
	5	5	5	5	3	3	3	3	3	3	3	3	3	3	3	3	2
Gly			1.06[c]	1.00[d]			1.01[c]										0.99
			1	1			1										1
Ala	1.03	1.01			1.00	1.05											2.01
	1	1			1	1											2
Val	2.95	2.96	2.99	2.96	1.97	1.94	2.03	1.92	1.96	2.02	1.95	1.96	1.81	1.93	1.93	1.84	
	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	
Ile	0.93	0.95	0.95	0.98													2.96
	1	1	1	1													3
Leu	5.15	5.11	5.09	5.13	5.00	4.89	5.25	3.97	3.93	4.13	4.02	3.90	4.15	4.09	4.07	4.29	5.03
	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	5
Nle													2.03	2.01	1.97	2.08	
													2	2	2	2	
Tyr	1.01	1.00	1.00	1.00	1.08	1.11	1.08	1.06	1.04	1.16	1.21	0.97	0.99	1.04	1.00	0.98	
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Phe								1.17	0.91	0.92	0.95	0.90	0.99	0.94	0.95	1.00	2.00
								1	1	1	1	1	1	1	1	1	2
His	2.85	3.00	3.03	3.02	2.93	2.85	2.97	3.03	2.95	3.21	3.04	3.01	2.79	2.95	2.99	2.98	3.99
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4
Lys	3.03	2.98	3.01	3.03	2.99	2.93	3.11	2.95	3.02	3.18	2.99	3.07	2.96	2.90	2.93	3.08	1.96
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2
Arg	1.94	1.94	1.73	1.73	1.95	1.94	2.06	1.95	1.95	2.06	1.94	1.98	1.94	1.92	1.94	1.99	2.92
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Met[e]	1.27	1.48	1.76	1.81	1.51	1.13	1.71	1.91	1.89	1.49	1.78	1.64					
	2	2	2	2	2	2	2	2	2	2	2	2					

[a] Values shown are the results obtained from amino acid analysis. [b] Expected values from amino acid analysis. [c] Aib substitutes Gly^{12}. [d] Pro substitutes Gly^{12}. [e] Met is labile under the hydrolysis conditions employed.

using an Applied Biosystems Model 430A peptide synthesizer and Version 1.2 of the software. Protection of the α-amino group was always *N*-butyloxycarbonyl (*N*-Boc). Side-chain protecting groups were as follows: N^{ϵ}-[[(2-chlorobenzyl)-oxy]carbonyl] for lysine; *O*-benzyl for glutamic acid, threonine, and serine; N^{in}-formyl for tryptophan; N^{G}-(*p*-toluenesulfonyl) for arginine; N^{π}-[(benzyloxy)methyl] for histidine. The synthesis commenced on 0.5 mmol of resin and continued, following the completion of resin-bound fully protected [Tyr^{34}]bPTH(23–34) or PTHrP(20–34) sequences, on a 0.25-mmol scale. This measure was undertaken to achieve better mixing and more efficient washings of the resin. All amino acid incorporation was undertaken by a double-coupling procedure. Preformed symmetrical anhydrides were used (1 mmol per coupling cycle) except for the coupling of the protected derivatives of Asn, Gln, His, and Arg, where preformed 1-hydroxybenzotriazole esters were used (2 mmol per coupling cycle). The completed side-chain-protected resin-bound peptide was cleaved from resin with simultaneous removal of side-chain protecting groups by the low/high-HF procedure (Tam et al., 1983). The crude peptide was extracted from the residue obtained from the cleavage reaction with 50% (v/v) acetic acid, loaded on a Sephadex G-50F column (95 × 5 cm), and eluted with 50% (v/v) acetic acid at a flow rate of 1 mL/min, 10-min fractions being collected. The crude lyophilzed product obtained from the column was purified by preparative reversed-phase high-performance liquid chromatography (HPLC) using a Waters Delta Prep 3000 system and a PrePak radial compression cartridge (30 × 5.7 cm) of Vydac protein C4-bonded silica partiles of 15–20 mm and 300-Å pore size. The preparative column was eluted with a linear gradient formed by increasing the percentage of buffer B in buffer A [buffer A = 0.1% (v/v) TFA in H_2O/CH_3CN, 95:5; buffer B = 0.1% (v/v) TFA in CH_3CN] at a flow rate of 100 mL/min. The fractions (0.1 min) collected from the preparative HPLC were analyzed on an analytical reversed-phase HPLC column (15 × 0.46 cm) packed with Vydac protein C4-bonded silica particles of 5 μm and pore size of 300 Å, the same solvent system as described above being employed, but at a flow rate of 1.5 mL/min. Peptide elution was monitored at 214 nm.

A battery of analytical tests was performed to assess peptide purity for each analogue (Caporale et al., 1989). These tests included analytical reversed-phase HPLC; amino acid analysis (following 70-h hydrolysis in constant-boiling HCl) using a Beckman amino acid analyzer; fast atom bombardment mass spectrometry (FAB-MS); amino acid sequencing using an Applied Biosystems Model 470A gas-phase sequencer, to confirm the amino acid sequence and to perform "preview" analysis to assess possible contamination by deletion-containing error peptides (Tregear et al., 1977); proton nuclear magnetic resonance spectrometry using a 400-MHz instrument, to assess the presence of low-abundance side products resulting from methionine oxidation, alkylation of aromatic residues, or backbone to side-chain rearrangements.

Results

Analysis of Peptides. The amino acid analyses of all analogues synthesized and used in this study are presented in Table I. Automated Edman sequence analysis (results not shown) confirmed the presence of the desired sequence in each case, and "preview" analysis indicated the absence of deletion-containing error peptides. By use of ^1H NMR, no side products were detected (data not shown). The purity of the synthetic peptide analogues was >98%, determined with data from the sequencing and analytical RP-HPLC.

Biological Properties of Peptides. Shown in Table II are bovine renal receptor binding and adenylate cyclase activities

Table II: Binding and Cyclase Activity of Agonist and Antagonist Analogues Derived from PTH and PTHrP Sequences with Bovine Renal Cortical Membranes

analogue	binding, K_b (nM)	cyclase	
		K_m (nM)	K_i (nM)
(I) [Tyr^{34}]hPTH(1–34)NH$_2$	0.7 ± 0.3	0.7 ± 0.1	
(1) [Ala^{12},Tyr^{34}]hPTH(1–34)NH$_2$	1.0 ± 0.04	1.5 ± 0.2	
(2) [D-Ala^{12},Tyr^{34}]hPTH(1–34)NH$_2$	0.8 ± 0.1	1.4 ± 0.1	
(3) [Aib^{12},Tyr^{34}]hPTH(1–34)NH$_2$	0.8 ± 0.1	0.6 ± 0.2	
(4) [Pro^{12},Tyr^{34}]hPTH(1–34)NH$_2$	590 ± 200	2400 ± 770	
(II) [Tyr^{34}]hPTH(7–34)NH$_2$	260 ± 40		840 ± 180
(5) [Ala^{12},Tyr^{34}]hPTH(7–34)NH$_2$	110 ± 30		410 ± 70
(6) [D-Ala^{12},Tyr^{34}]hPTH(7–34)NH$_2$	120 ± 10		610 ± 120
(7) [Pro^{12},Tyr^{34}]hPTH(7–34)NH$_2$	470 ± 40		1400 ± 670
(III) [Tyr^{34}]bPTH(7–34)NH$_2$	80 ± 10		880 ± 70
(8) [Sar^{12},Tyr^{34}]bPTH(7–34)NH$_2$	500 ± 90		2500 ± 730
(9) [Aib^{12},Tyr^{34}]bPTH(7–34)NH$_2$	50 ± 10		550 ± 140
(10) [β-Ala^{12},Tyr^{34}]bPTH(7–34)NH$_2$	140 ± 20		300 ± 70
(11) [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	10 ± 1		70 ± 10
(12) [Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	110 ± 10		740 ± 110
(IV) [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH$_2$	150 ± 10		1550 ± 330
(13) [Nle^{8,18},D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	20 ± 1		130 ± 10
(14) [Nle^{8,18},D-α-Nal^{12},Tyr^{34}]bPTH(7–34)NH$_2$	10 ± 1		350 ± 70
(15) [Nle^{8,18},D-β-Nal^{12},Tyr^{34}]bPTH(7–34)NH$_2$	10 ± 2		140 ± 40
(16) [Nle^{8,18},Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	50 ± 3		510 ± 50
(V) PTHrP(7–34)NH$_2$	260 ± 40		610 ± 130
(17) [D-Trp^{12}]PTHrP(7–34)NH$_2$	40 ± 10		110 ± 10

of reference analogues in the agonist (I) and antagonist series (II–V) and position 12 substituted analogues of PTH (peptides **1–16**) and PTHrP (peptide **17**).

The K_b and K_m of agonist analogues containing Ala^{12}, D-Ala^{12}, and Aib^{12} (**1–3**) are in the range of 0.7–1.0 nM and 0.6–1.5 nM, respectively. These values are similar to those obtained for the parent agonist I (0.7 nM). The Pro^{12} analogue (**4**) is approximately 840- and 3500-fold less active than [Tyr^{34}]hPTH(1–34)NH$_2$ (I) in binding and adenylate cyclase assays, respectively.

In the antagonist series related to the parent analogues II–IV, analogues [Ala^{12},Tyr^{34}]- and [D-Ala^{12},Tyr^{34}]hPTH(7–34)NH$_2$, **5** and **6**, respectively, were about twice as potent as the parent peptide II in both binding and cyclase assays. Analogues **9** and **10**, modified at position 12 by Aib and β-Ala, respectively, were 2–3 times more potent in inhibiting agonist-stimulated (3 nM [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$) adenylate cyclase activity than the corresponding parent antagonist III. However, analogue **10** displays lower binding affinity than the parent peptide III (cf. K_b = 140 and 80 nM, respectively). This finding is in contrast to the generally observed parallel changes in inhibition of PTH-stimulated adenylate cyclase and binding affinity for the rest of the position 12 modified analogues. The reason for this discrepancy is not known. Both imino acid containing analogues, **7** and **8**, in the antagonist series (containing Pro^{12} and Sar^{12}, respectively) display approximately one-half to one-sixth the binding affinity (470 and 500 nM for **7** and **8**, respectively, compared to 260 and 80 nM for II and III, respectively) and about 2–3-fold diminished adenylate cyclase inhibitory potency compared to the parent antagonists II and III (1400 and 2500 nM for **7** and **8**, respectively, and 840 and 880 nM for II and III, respectively). Replacement of Gly^{12} with D-Trp in III resulted in antagonist **11**, which was 10-fold more potent in the binding assay (6.7 nM) and about 12-fold more potent at inhibiting agonist-induced adenylate cyclase activity (69 nM). In contrast, introduction of L-Trp^{12} into III (antagonist **12**) failed to alter either binding affinity or inhibition of adenylate cyclase activity compared to the parent peptide. The combination of Nle^{8,18} and either D-Trp (**13**) or D-α/β-naphthylalanine (**14** and **15**, respectively) in position 12 also generated highly potent antagonists. Antagonists **13–15** were 10–14 times more potent in binding and 5–12 times more potent as inhibitors of adenylate cyclase than the corresponding parent antagonist IV. In PTHrP(7–34)NH$_2$ (V), substitution of Gly^{12} with D-Trp (analogue **17**) yielded a peptide with 6-fold greater affinity and inhibitory potency than its parent structure.

Table III: Binding and Cyclase Activity of Antagonist Analogues Derived from bPTH(7–34)NH$_2$ with Bone-Derived ROS17/2.8 Cells

analogue	binding, K_b (nM)	cyclase, K_i (nM)
(III) [Tyr^{34}]bPTH(7–34)NH$_2$	700 ± 90	2700 ± 520
(11) [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	120 ±	210 ± 120
(12) [Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	400 ± 20	1360 ± 80
(IV) [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH$_2$	960 ± 170	1550 ± 360
(13) [Nle^{8,18},D-Trp^{12},Tyr^{34}]-bPTH(7–34)NH$_2$	180 ± 30	70 ± 20
(16) [Nle^{8,18},Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	410 ± 30	220 ± 30

Table III summarizes data for receptor binding and inhibition of PTH-stimulated adenylate cyclase activity for selected analogues in the bone-derived system. Reference analogues III and IV are compared to D- or L-Trp^{12}-substituted analogues of PTH (peptides **11–13** and **16**). Substitution of Gly^{12} with D-Trp enhances binding about 5-fold (see Table III, **11** versus III and **13** versus IV) compared to the 10-fold enhancement observed in the renal-based assay (see Table II, same analogues). The effect of this substitution on inhibition of PTH-stimulated adenylate cyclase activity in bone-derived cells is comparable to that observed in the renal membranes. Substitution by L-Trp^{12} resulted in a 2-fold increase in binding and inhibitory potency in the bone system, comparable to the effect observed for the same replacement in [Nle^{8,18},Tyr^{34}]bPTH-(7–34)NH$_2$ in the renal assays.

For agonists and antagonists, a close correlation between rank order of potency in the binding and adenylate cyclase assays is apparent. Furthermore, in the agonist series the K_b and K_i for each analogue correspond closely. For the antagonist series, however, a consistently weaker antagonist potency (about 1 order of magnitude) is observed for the K_i versus binding affinity, K_b. The reason for this discrepancy is not known.

A 3-fold difference in receptor avidity was observed for antagonists derived from human sequence versus bovine sequence (cf. II and III in Table II). Therefore, we evaluated

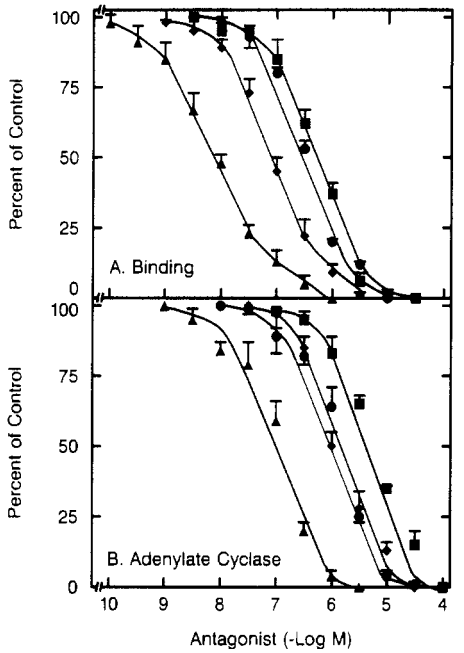

FIGURE 3: Effects of PTH and PTHrP analogues on (A) binding of radiolabeled PTH ligand, [Nle^{8,18},[^{125}I]iodo-Tyr^{34}]bPTH(1–34)NH_2, and (B) inhibition of PTH-stimulated adenylate cyclase in bovine renal cortical membranes. Assays were performed as described under Materials and Methods. Analogues: [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH_2 (▲); [Tyr^{34}]bPTH(7–34)NH_2 (◆); PTHrP(7–34)NH_2 (●); [Sar^{12},Tyr^{34}]bPTH(7–34)NH_2 (■).

the effects of introducing modifications into both sequences. Binding and dose–response curves for PTHrP(7–34)NH_2 and [Tyr^{34}]bPTH(7–34)NH_2 and some of its analogues modified at position 12 are depicted in Figure 3.

Discussion

In order to design more potent antagonists of PTH and PTHrP, it is necessary to identify sites within the hormone molecule for which structural modification can be used to enhance affinity for the receptor without restoring agonist-like properties or diminishing specificity of receptor interaction. There are two principal ways to accomplish this objective: (1) stabilize an antagonist in a conformation favored by the receptor or (2) introduce new structural moieties that will add binding elements that interact with the receptor at sites different from those present in the native agonist. These novel binding elements should not disrupt the "bioactive conformation" required for receptor binding.

Position 12 in the PTH sequence lies outside the previously identified N-terminal "receptor-activation domain" (Rosenblatt, 1986). The studies described in this paper demonstrate that, with regard to adenylate cyclase linked receptor activity, glycine at position 12 is not essential. This position, in both a series of agonists and antagonists, tolerates a wide variety of substitutions (cf. analogues **1**, **2**, **4–6**, **9**, and **10**). The only substitutions demonstrated to diminish biological activity were the N-substituted amino acids, namely, Pro (analogues **3** and **7**) and Sar (analogue **8**). These findings suggest that a wide range of conformational latitude is tolerated in the region neighboring position 12, especially among residues accommodated by α-helix or β-sheet. However, known N-alkylated amino acids, which may act as helix breakers, are poorly tolerated.

This wide range of tolerance for Gly^{12} substitution is unusual and contrasts with structure–activity studies in other hormonal systems, such as cholecystokinin (Fournie-Zaluski et al., 1985), substance P (Blumberg & Teichberg, 1981), enkephalin (Walker et al., 1977), and LH-RH (Vale et al., 1977; Perrin et al., 1980), where substitutions of a wide range of amino acids at a particular site are not well tolerated.

Enhancement of hydrophobicity within the binding domain of a hormone has increased bioactivity in several hormonal systems (Manning et al., 1982a,b; Rees et al., 1974; Rivier & Vale, 1978; Fries et al., 1982; Engberg et al., 1981). Therefore, we attempted to increase antagonist activity by increasing hydrophobicity at position 12. Such modifications follow the rationale of introducing new potential receptor-binding elements into the antagonist sequence.

Analogues containing D-Trp, L-Trp, or D-α- or D-β-Nal residues in position 12 of either [Tyr^{34}]bPTH(7–34)NH_2 (III) or [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH_2 (IV) were prepared (analogues **11–16**) and evaluated. Replacement of Gly^{12} in III by D-Trp produced the highly potent antagonist **11**, which was 12-fold more potent than III in binding and inhibition of PTH-stimulated adenylate cyclase. The similar potencies of analogues substituted by either D-Trp^{12} or D-α- and D-β-naphthylalanyl residues are in accord with observations of others regarding the functional equivalence of the indolyl and naphthyl moieties (Nestor et al., 1982, 1984). The similar potencies observed for analogues **14** and **15** containing D-α-Nal and D-β-Nal, respectively, suggest that additional hydrophobic interactions can be accommodated within a complementary domain in the receptor. This enhanced binding, however, does not extend to the increased hydrophobicity present in the original stereochemistry because the increased antagonistic properties of D-Trp^{12} substitution are lost by introducing L-Trp^{12}. This may indicate that introduction of highly hydrophobic side chains in the D configuration at position 12 provides auxillary hydrophobic interactions with the receptor that result in enhanced binding.

Along similar lines, incorporation of D-Trp in position 12 of PTHrP(7–34)NH_2 (V) to yield analogue **17** generated a more potent antagonist of PTHrP and reduced the partial agonism that had been observed previously in the parent antagonist (McKee et al., 1988; R. McKee, unpublished data). The disappearance of partial agonism may be attributed to the auxillary hydrophobic interaction with receptor, discussed above, which may impede assumption of a conformation required for receptor activation.

A comparison between properties observed in bovine renal cortical membranes and bone-derived rat osteosarcoma cells (ROS17/2.8) demonstrates, qualitatively, that the potency enhancement resulting from introduction of D-Trp^{12} is similar across the two systems (Tables II and III). These findings strengthen the concept that D-Trp substitution is advantageous in terms of receptor interaction because the observations are consistent across target tissues (renal vs bone), species (bovine vs rat), and nature of the preparations (membranes vs intact cells).

In conclusion, on the basis of conformational predictions, we explored the biological effects of structural modification of PTH and PTHrP at position 12. We demonstrated that position 12 in PTH tolerates a wide range of structural latitude (excluding *N*-methyl amino acids) without substantial weakening of receptor interaction. These findings permitted us to select this site for introduction of hydrphobic amino acids not present in the native PTH or PTHrP sequences. The introduction of D-Trp or other more hydrophobic (D-α/β-Nal) residues at position 12 enhances antagonist activity for analogues of the calcium-mobilizing hormones. These analogues are active in the low nanomolar range. Extension of this approach may lead to more potent PTH and PTHrP antag-

onists and, when applied to other bioactive peptides, may provide a promising direction for the rational design of potent antagonists.

Added in Proof

After submission of this paper, a publication on the structure of PTHrP(1–34) determined by NMR appeared (Barden & Kemp, 1989). The authors predict a compact structure for the 1–34 peptide with a reverse turn, formed by residues 10–13, which they also predict to be present in PTH(1–34). The authors also present data to show that PTHrP(7–34) is less structured. This decrease in structure may account for the ability of hydrophobic substitutions at position 12 to increase affinity for the receptor by permitting interaction with a novel site(s) on the receptor.

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References

Aloj, S., & Edelhoch, H. (1972) *Arch. Biochem. Biophys. 150*, 782.

Barden, J. A., & Kemp, B. E. (1989) *Eur. J. Biochem. 184*, 379.

Bluberg, S., & Teichberg, V. I. (1981) *Biochem. Biophys. Res. Commun. 99*, 752.

Brewer, H. B., Jr., Farwell, T., Rittel, W., Littledike, T., & Arnaud, C. D. (1974) *Am. J. Med. 56*, 759.

Bundi, A., Andreatta, R. H., & Wutrich, K. (1978) *Eur. J. Biochem. 91*, 201.

Caporale, L., Nutt, R., Levy, J., Smith, J., Arison, B., Bennett, C., Albers-Schonberg, G., Pitzenberger, S., Rosenblatt, M., & Hirschmann, R. F. (1989) *J. Org. Chem. 54*, 343.

Cheng, Y.-C., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099.

Chon, D. V., & MacGregor, R. R. (1981) *Endocr. Rev. 2*, 1.

Chou, P. Y., & Fasman, G. D. (1978) *Adv. Enzymol. 47*, 45.

Doppelt, S. H., Neer, R. M., Nussbaum, S. R., Federico, P., Potts, J. T., Jr., & Rosenblatt, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7557.

Engberg, G., Svensson, T. H., Rosell, S., & Folkers, K. (1981) *Nature 293*, 222.

Epand, R. M., Epand, R. F., Hui, S. W., He, N. B., & Rosenblatt, M. (1985) *Int. J. Pept. Protein Res. 25*, 594.

Fournie-Zaluski, M.-C., Belleney, J., Durieux, C., Gacel, G., Roques, B. P., Begue, D., Menant, I., Lux, B., & Gerard, D. (1985) *Ann. N.Y. Acad. Sci. 448*, 598.

Fries, J. L., Murphy, W. A., Sueiras-Daiz, J., & Coy, D. H. (1982) *Peptides 3*, 811.

Fukayama, S., Bosma, T. J., Goad, D. L., Voelkel, E. F., & Tashjian, A. H., Jr. (1988) *Endocrinology 123*, 2841.

Goldman, M. E., Chorev, M., Reagan, J. E., Nutt, R. F., Levy, J. J., & Rosenblatt, M. (1988a) *Endocrinology 123*, 1468.

Goldman, M. E., McKee, R. L., Caulfield, M. P., Reagan, J. E., Levy, J. J., Gay, C. T., DeHaven, P. A., Rosenblatt, M., & Chorev, M. (1988b) *Endocrinology 123*, 2597.

Horiuchi, N., Rosenblatt, M., Keutmann, H. T., & Potts, J. T., Jr. (1983a) *Am. J. Physiol. 244*, E589.

Horiuchi, N., Holick, M. F., Potts, J. T., Jr., & Rosenblatt, M. (1983b) *Science 220*, 1053.

Horiuchi, N., Caulfield, M. P., Fisher, J. E., Goldman, M. E., McKee, R. L., Reagan, J. E., Levy, J. J., Nutt, R. F., Rodan, S. B., Schofield, T. L., Clemens, T. L., & Rosenblatt, M. (1987) *Science 238*, 1566.

Juppner, H., Abou-Samra, A.-B., Uneno, S., Gu, W.-X., Potts, J. T., Jr., & Segre, G. V. (1988) *J. Biol. Chem. 263*, 8557.

Kemp, B. E., Moseley, J. M., Rodda, C. P., Ebelling, P. R., Wettenhall, E. H., Stapleton, D., Diefenbach-Jagger, H., Ure, F., Michelangeli, V. P., Simmons, H. A., Raisz, L. G., & Martin, T. J. (1987) *Science 238*, 1568.

Kyte, T., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105.

Mahaffey, J. E., Rosenblatt, M., Shepard, G. L., & Potts, J. T., Jr. (1979) *J. Biol. Chem. 254*, 6496.

Manning, M., Olma, A., Klis, W. A., Kolodziejczyk, M., Seto, J., & Sawyer, W. H. (1982a) *J. Med. Chem. 25*, 45.

Manning, M., Klis, W. A., Olma, A., Seto, J., & Sawyer, W. H. (1982b) *J. Med. Chem. 25*, 414.

McKee, R. L., Goldman, M. E., Caulfield, M. P., DeHaven, P. A., Levy, J. J., Nutt, R. F., & Rosenblatt, M. (1988) *Endocrinology 122*, 3008.

Merrifield, R. B. (1969) *Adv. Enzymol. Relat. Subj. Biochem. 32*, 221.

Moseley, J. M., Kubota, M., Diefenbach-Jagger, H. D., Wettenhall, R. E. H., Kemp, B. E., Suva, L. J., Rodda, C. P., Ebeling, P. R., Hudson, P. J., Zajac, C. D., & Martin, T. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5048.

Nestor, J. J., Jr., Ho, T. L., Simpson, R. H., Horner, B. L., Jones, G. H., McRae, G. I., & Vickery, B. H. (1982) *J. Med. Chem. 25*, 795.

Nestor, J. J., Jr., Tahilramani, R., Ho, T. L., McRae, G. I., & Vickery, B. H. (1984) *J. Med. Chem. 27*, 1170.

Perrin, M. H., Rivier, J. E., & Vale, W. V. (1980) *Endocrinology 106*, 1289.

Rabbani, S. A., Mitchell, J., Roy, D. R., Hendy, G. N., & Goltzman, D. (1988) *Endocrinology 123*, 2709.

Rees, R. W. A., Foell, J. J., Chai, S.-Y., & Grant, N. (1974) *J. Med. Chem. 17*, 1016.

Rivier, J., & Vale, W. (1978) *Life Sci. 23*, 869.

Rodan, S. B., Insogna, K. L., Vignery, A. M., Steward, A. F., Broadus, A. E., D'Sousa, S. M., Bertolini, D. R., Mundy, G. R., & Rodan, G. A. (1983) *J. Clin. Invest. 72*, 1511.

Rosenblatt, M. (1986) *N. Engl. J. Med. 315*, 1004.

Rosenblatt, M., Goltzman, D., Keutmann, H. T., Tregear, G. W., & Potts, J. T., Jr. (1976) *J. Biol. Chem. 251*, 159.

Rosenblatt, M., Callahan, E. N., Mahaffey, J. E., Pont, A., & Potts, J. T., Jr. (1977) *J. Biol. Chem. 252*, 5847.

Rosenblatt, M., Segre, G. V., Tyler, G. A., Shepard, G. L., Nussbaum, S. R., & Potts, J. T., Jr. (1980) *Endocrinology 107*, 545.

Ross, A. M., & Golub, E. E. (1988) *Nucleic Acids Res. 16*, 1801.

Segre, G. V., Tully, G. L., III, Rosenblatt, M., Laugharn, J. A., Reit, B., & Potts, J. T., Jr. (1979) *Calcif. Tissue Int. 28*, 171 (Abstract).

Shah, G. V., Epand, R. M., & Orlowski, R. C. (1987) *Mol. Cell. Endocrinol. 49*, 203.

Stewart, A. F., Insogna, K. L., Goltzman, D., & Broadus, A. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1454.

Stewart, A. F., Wu, T., Goumas, D., Burtis, W. J., & Broadus, A. E. (1987) *Biochem. Biophys. Res. Commun. 146*, 672.

Strewler, G. J., Williams, J., & Nissenson, R. A. (1983) *J. Clin. Invest. 71*, 769.

Strewler, G. J., Stern, P. H., Jacobs, J. W., Eveloff, J., Klein, R. F., Leung, S. C., Rosenblatt, M., & Nissenson, R. A. (1987) *J. Clin. Invest. 80*, 1803.

Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Difenbach-Jagger, H., Rodda, C. P., Kemp B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J., & Wood, W. I. (1987) *Science 238*, 893.

Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442.
Tregear, G. W., van Reitschoten, J., Sauer, R., Niall, H. D., Keutmann, H., & Potts, J. T., Jr. (1977) *Biochemistry 16*, 2817.
Vale, W., Rivier, C., Brown, M., & Rivier, J. (1977) in *Hypothalamic Peptide Hormones and Pituitary Regulation* (Porter, J. C., Ed.) p 123, Plenum Press, New York.
Walker, J. M., Bernston, G. G., Sandman, C. A., Coy, D. H., Schally, A. V., & Kastin, A. J. (1977) *Science 196*, 85.

A Model for the Interaction of Muscarinic Receptors, Agonists, and Two Distinct Effector Substances

Allen P. Minton[‡] and Mordechai Sokolovsky*[,§]

Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Laboratory of Neurobiochemistry, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel Aviv 69978, Israel

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ABSTRACT: The binding of the agonist carbamylcholine to muscarinic receptors in rat heart myocytes from young and aged cultures and in rat atrial membranes has been measured in the absence and presence of GppNHp, pertussis toxin, and/or batrachotoxin. The effect of each of the added substances upon agonist binding was accounted for by a model according to which the receptor may form an equilibrium complex with agonist and either of two distinct effector substances, one of which is postulated to increase the affinity of receptor for agonist and the other of which is potulated to decrease the affinity of receptor for agonist.

While it is known that the binding of muscarinic agonists to their receptors inhibits the production of cyclic AMP, presumably through interaction with the adenylate cyclase system (Watanabe et al., 1978; Brown, 1979; Van Sande et al., 1977; Olianas et al., 1983), the molecular mechanism of interaction between muscarinic receptors and other membrane and intracellular components such as ion channels is as yet unclear [for reviews, see McKinney and Richelson (1984), Nathanson (1987), Sokolovsky et al. (1986), and Sokolovsky (1989)]. Because the addition of GTP and GTP analogues to membrane preparations has a significant effect on the binding of muscarinic agonists to receptors, the existence of agonist-mediated interactions between muscarinic receptors and one or more forms of G-protein is inferred (McKinney & Richelson, 1984; Nathanson, 1987; Sokolovsky et al., 1986; Sokolovsky, 1989). While classical antagonist binding isotherms are generally explicable in terms of a model for the binding of ligand to a single class of homogeneous sites (Nathanson, 1987; Sokolovsky, 1989), data characterizing the displacement of antagonist by agonist have usually required that two or three affinity classes of binding sites be postulated (Birdsall et al., 1980). Common to both the two- and three-affinity-class models has been the assumption that the fractional abundance of each class of site is independent of the concentration of agonist. The binding isotherm of agonist to n affinity classes of sites is then specified by n equilibrium association or dissociation constants and $n - 1$ fractional site abundances.

In previous studies of the effect of exogenously added substances (such as GppNHp)[1] on the affinity of receptor for agonist, the results have typically been analyzed by fitting a phenomenological two- or three-site-class model to the data (Berrie et al., 1979; Sokolovsky et al., 1980; Wei & Sulakhe, 1980; Burgisser et al., 1982; Ehlert et al., 1981; Harden et al., 1982; Walbroeck et al., 1982). If affinity for agonist is increased in the presence of the additive, the effect is "explained" as an additive-induced conversion of lower affinity sites to higher affinity sites; conversely, if affinity for agonist is decreased in the presence of the additive, the effect is "explained" as an additive-induced conversion of higher affinity sites to lower affinity sites (Birdsall et al., 1980; Sokolovsky et al., 1986). We feel that such "explanations" are merely parameterized descriptions of the data and provide little or no insight into the molecular mechanism(s) underlying the effect of exogenous additives upon agonist binding. The purpose of the present work is to present a mechanistic model that attempts to describe agonist binding and the effects of exogenous additives on agonist binding *in the context of receptor function*. Such a model must differ from a phenomenological description of agonist binding in at least two important ways:

(1) When phenomenological two- or three-site-class models are fit to a series of isotherms characterizing agonist binding to receptor in the presence of varying amounts of exogenous additive A, it is commonly observed that the affinities for agonist, as well as the abundances of the apparent site classes, vary substantially with the concentration of A (Sokolovsky et al., 1986; Sokolovsky, 1989). In contrast to a phenomenological approach to the definition of site classes, we adopt a mechanistic approach wherein total receptor is considered to exist as a population of substates. An individual substate of receptor may differ from other substates by virtue of intrinsic differences in polypeptide structure or by virtue of covalent or noncovalent association with other naturally occurring or

* Author to whom correspondences and reprint requests should be addressed.
‡ National Institutes of Health.
§ Tel-Aviv University.

[1] Abbreviations: BTX, batrachotoxin; 4NMPB, *N*-methyl-4-piperidyl benzilate; GppNHp, guanyl-5′-yl imidodiphosphate; PT, pertussis toxin.

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