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In vivo STUDIES ON AN ANTAGONIST OF PARATHYROID HORMONE [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)AMIDE

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1 The actions of parathyroid hormone (PTH) are antagonized *in vitro* by the peptide [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide, an analogue of PTH. In this paper, the actions of the inhibitory peptide were investigated *in vivo*.

2 Native parathyroid hormone (bPTH-(1-84)), administered i.v. (0.17–1.51 nmol in a volume of 0.3 ml) to 7 day old chicks produced hypercalcaemia but administration of the analogue in doses up to 173 nmol was ineffective in this respect.

3 The analogue failed to antagonize the hypercalcaemia produced by bPTH-(1-34) when injected, in 10 fold molar excess, 2 min before or simultaneously with bPTH-(1-34).

4 Normocalcaemia was restored in parathyroidectomized rats by intravenous infusion of bPTH-(1-84) at 32 pmol $kg^{-1} h^{-1}$. Addition of the analogue to the infusion fluid in a 200 fold molar excess did not affect the concentrations of calcium and phosphate in the plasma, cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in the urine or phosphate clearance but produced a significant ($P < 0.05$) rise in urinary calcium clearance.

5 The results suggest that the peptide [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide does not antagonize the actions of PTH *in vivo* and demonstrate an important dichotomy between *in vitro* and *in vivo* biological properties of the PTH analogue.

Introduction

The actions of parathyroid hormone (PTH) *in vivo* are antagonized by the peptide [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide, an analogue of the naturally occurring hormone. In the canine renal adenylate cyclase assay, the peptide caused 50% inhibition of PTH-stimulated adenylate cyclase when present at concentrations equimolar to PTH, and, in higher concentrations, it abolished the response (Rosenblatt, Callahan, Mahaffey, Pont & Potts, 1977). Competitive binding studies using a renal radioreceptor assay showed that the inhibitory properties of this analogue resulted directly from its occupation of PTH-specific binding sites (Segre, Rosenblatt, Reiner, Mahaffey & Potts, 1979a). A binding constant equal to that of native bPTH-(1-84) was also demonstrated for the analogue. Similarly, the analogue inhibited PTH-stimulated increases in cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in intact living human cells derived from bone or skin (Goldring, Mahaffey, Rosenblatt, Dayer, Potts & Krane, 1979) and had an apparent binding constant in these systems equal to that observed in the renal membrane system. In order to determine its potential as an antagonist of PTH *in vivo*, the activity of [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide was examined by studying its effects on (a) acute PTH-induced hypercalcaemia in the chick, which is predominantly due to the effect of PTH on bone (Parsons, Reit & Robinson, 1973), and (b) on multiple parameters of PTH action during continuous infusion to rats with indwelling cannulae.

Methods

Chicks

Blood samples for calcium analysis were taken from groups of six to eight 7–10 day old male chicks (Rhode Island Red/Light Sussex Cross), in the hypercalcaemia assay described previously (Parsons *et al.*, 1973), 1 h after intravenous injection (0.3 ml) of the analogue or of PTH or a combination of both. The effects of the two peptides were also observed in birds injected intraperitoneally with 5 μCi ^{47}Ca (in 0.2 ml) 3 days before the assay.

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Rats

Male Wistar rats, weighing 300–350 g, were parathyroidectomized by cautery and a blood sample taken from each by cardiac puncture after 24 h. Animals were excluded from the study if they had a plasma calcium value of 1.63 mmol/l (6.5 mg/100 ml) or above. After parathyroidectomy, a group of 12 rats was prepared with indwelling venous catheters as previously described, with harness and swivels allowing the animals relatively unrestricted movement (Stevenson, Tsakok & Parsons, 1980). Rats were then placed in metabolism cages and infused for 5 days with bPTH-(1-84) at 10.5 pmol/h (0.1 μg/h) the rate previously shown to restore normocalcaemia of 2.18 ± 0.11 mmol/l (8.71 ± 0.43 mg/100 ml) (Stevenson & Parsons, unpublished observation). At 09 h 00 min on day 6, the analogue [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide was added to the infusion solution of six of the rats (randomly selected) in 200 fold molar excess. At 15 h 00 min the infusion fluid was replaced by a freshly prepared solution containing bPTH-(1-84) and the analogue. The infusion was continued until 09 h 00 min on day 7. Separate urine collections were made during each of the above three periods.

Measurements

Plasma samples (0.1 ml) were diluted 1:10 with water (automatic diluter) and deproteinized by addition of 1 ml 6% (v/v) perchloric acid containing 0.6% (w/v) lanthanum chloride. These samples were then analysed for calcium by atomic absorption on a Pye Unicam Atomic Absorption Spectrophotometer. Urine calcium was measured by atomic absorption and by fluorimetry using the Corning Calcium Analyzer Model 940, these two independent methods being employed to verify the significant increase in urinary calcium excretion in the second collection period. Plasma phosphate was analysed by the indirect molybdenum atomic absorption method of Parsons, Dawson, Callahan & Potts (1970). Plasma and urine creatinine was measured by the method of Hare (1950). Urine was assayed for cyclic AMP with a radioimmunoassay kit (from the Radiochemical Centre, Amersham) which employs adenosine 3',5'-cyclic phosphate as standard and [8-^{3}H]-adenosine 3',5'-cyclic phosphate as tracer. All samples were measured in the same assay which has an intra-assay coefficient of variation of $< 7\%$.

Results

Injection to chicks

Intravenous injection of bPTH-(1-84) to 7 day old chicks at doses of 0.17–1.51 nmol (1.6–14.4 μg; 3–27 u) produced increases in the plasma calcium concentration of up to 0.5 mM (2 mg/100 ml) within 1 h, but the analogue [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide (2.6–173.5 nmol, i.e. 10–680 μg) was ineffective in this respect (Figure 1). In contrast to bPTH-(1-84), [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide did not cause the mobilization of ^{47}Ca from bone. When injected in a ten fold molar excess, 2 min before or simultaneously with 1.3 nmol (5.4 μg)

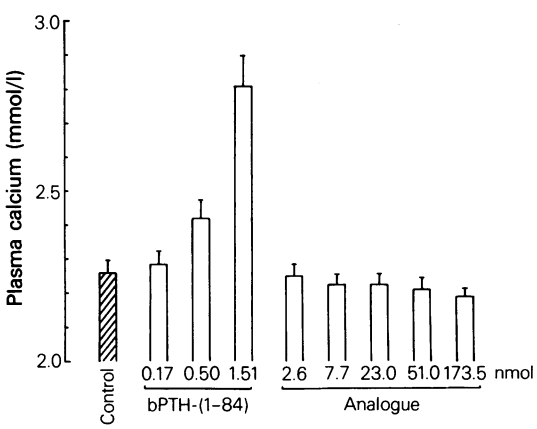

Figure 1 Plasma calcium concentrations in 7 day old chicks 60 min after intravenous injection of bPTH-(1-84) or the analogue [Nle-8, Nle-18, Tyr-34]-bPTH-(3-34)amide. Hatched column, vehicle-treated controls; open columns, drug-treated. Each column is the mean of 8 determinations and is shown with the standard error.

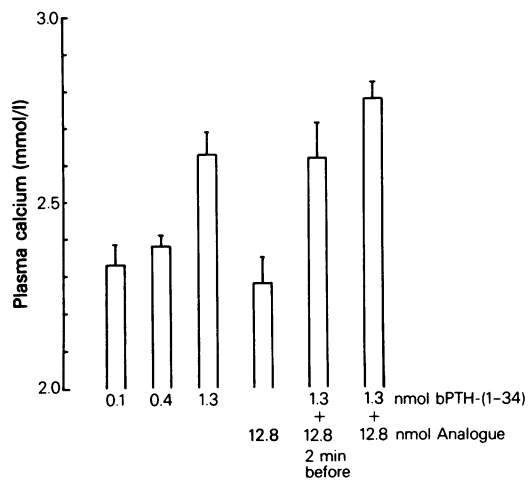

Figure 2 Plasma calcium concentrations in 7 day old chicks 60 min after intravenous injection (a) of bPTH-(1-34) or the analogue alone, or (b) of the analogue 2 min before or simultaneously with bPTH-(1-34). Each column is the mean of 6 determinations and is shown with its standard error.

bPTH-(1-34), the analogue did not affect the response to bPTH-(1-34) (Figure 2).

Infusion to rats

As described above, the infusion rate of bPTH-(1-84) found to restore normocalcaemia to parathyroidectomized rats in this weight range was 10.5 pmol/h (0.1 μg/h). The effects of adding the analogue, in a 200 fold molar excess, to the infusion fluid on the 6th day are shown in Figure 3. Diurnal changes in plasma calcium and phosphate were evident but there were no significant differences in the plasma concentrations between the two groups. However, there was significantly ($P<0.01$) greater urinary clearance of calcium during the period 6–12 h after the addition of analogue (Table 1). No significant difference in creatinine clearance was observed between the two groups at each collection period. The urinary calcium values obtained by atomic absorption were consistently 9% lower than by fluorimetry. Values for urinary cyclic AMP and phosphate clearance were extremely variable and no significant difference was observed between groups (Table 1).

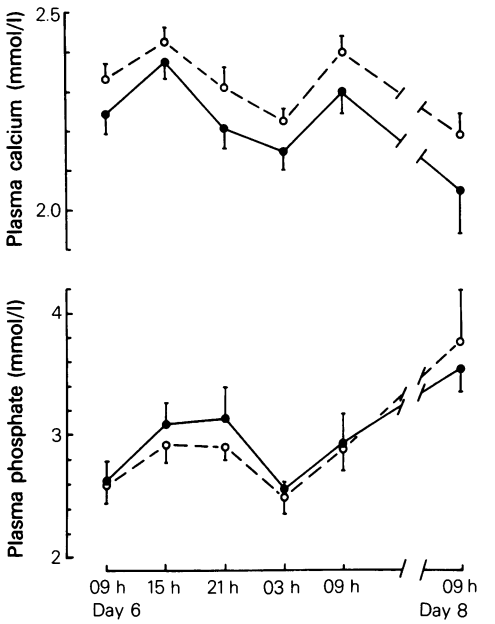

Figure 3 Plasma calcium and phosphate concentrations in parathyroidectomized rats during infusion of bPTH-(1-84) alone at 10 pmol/h (●) or in combination with the analogue at 200 fold molar excess (○). All infusions were stopped at 09 h 00 min on day 7. Each point is the mean of 6 determinations and is shown with its standard error.

Discussion

The results of these *in vivo* experiments with the PTH analogue [Nle-8, Nle-18, Tyr-34]bPTH-(3-34)amide are difficult to reconcile with the biological properties previously documented *in vitro*. The

Table 1 Urine clearance of calcium, phosphate and cyclic AMP in 2 groups of six parathyroidectomized rats on day 6 of intravenous infusion of either bPTH-(1-84) alone at 10.5 pmol/h or of bPTH-(1-84) plus the analogue at 200 fold molar excess

			09 h 00 min–15 h 00 min	*15 h 00 min–21 h 00 min*	*03 h 00 min–09 h 00 min*
$\frac{\text{Calcium clearance}}{\text{Creatinine clearance}} \times 10^{-3}$	bPTH-(1-84)	Fluorimetry	12.02 ± 3.12	9.19 ± 1.53	7.30 ± 1.71
		Atomic absorption		8.35 ± 1.32	
	bPTH-(1-84) + analogue	Fluorimetry	12.44 ± 1.39	16.55 ± 1.74**	10.41 ± 1.20
		Atomic absorption		15.09 ± 1.56**	
$\frac{\text{Phosphate clearance}}{\text{Creatinine clearance}} \times 10^{-3}$	bPTH-(1-84)		43.7 ± 24.9	63.2 ± 19.5	70.9 ± 21.2
	bPTH-(1-84) + analogue		49.1 ± 14.7	80.4 ± 8.9	60.5 ± 9.1
Cyclic AMP clearance (nmol/100 ml glomerular filtrate)	bPTH-(1-84)		0.93 ± 0.17	1.04 ± 0.19	1.12 ± 0.21
	bPTH-(1-84) + analogue		1.00 ± 0.18	0.97 ± 0.05	1.13 ± 0.17

Results are mean ± s.e.mean. Significant differences between results of infusing bPTH-(1-84) and bPTH-(1-84) plus analogue are represented by asterisks (** $P<0.01$)

analogue is a potent competitive and specific inhibitor of PTH action devoid of PTH-like agonist activity when tested in the renal (Rosenblatt *et al.*, 1977) and in the skeletal systems (Goldring *et al.*, 1979). However, our investigation failed to reveal antagonism of PTH action in the chick hypercalcaemia assay (Figure 2). The only effect displayed by the analogue in a PTH-infused parathyroidectomized rat model which we developed for these studies was to increase urinary calcium excretion (Table 1). This increase in calcium clearance, observed between 6 and 12 h after addition of the analogue to the bPTH-(1-84) solution, was confirmed when the urine samples were reanalysed by fluorimetry. It was not accompanied by a significant change in levels of cyclic AMP appearing in the urine. However, our studies do not exclude a possible inhibition of PTH-stimulated increases in cyclic AMP occurring intracellularly in renal cells as might be expected from the *in vitro* effects of the analogue on cyclic AMP levels (Rosenblatt *et al.*, 1977; Goldring *et al.*, 1979). Several arguments can be offered to explain the failure of the synthetic hormone analogue to demonstrate inhibitory properties *in vivo* and the consequent discrepancy between findings *in vivo* and *in vitro*:

It seems unlikely that the analogue is destroyed *in vivo* more rapidly than native parathyroid hormone, for the studies in rats in which no antagonism was evident, were carried out at a 200 fold molar excess of analogue to bPTH-(1-84). Hence, if degradation alone were to account for the lack of activity *in vivo*, the analogue must be assumed to have a half life of only a few seconds in the circulation ($T_{1/2}$ of bPTH-(1-84) is approximately 4 min). This explanation seems improbable, since very few peptides have a circulating half life shorter than one circulation time. Another explanation for the low inhibitory potency of the analogue may be that the substitution of methionine by norleucine reduces biological activity *in vivo* as suggested by earlier studies on D-Tyr-34 analogues (Rosenblatt, Coltrera, Shepard, Gray, Parsons & Potts, 1981).

On the other hand, it is possible that not all of the actions of PTH involved in maintaining normocalcaemia are mediated via stimulation of the enzyme adenylate cyclase (see Goltzman, 1979). In this event, the inhibitory properties of the analogue with respect to adenylate cyclase activity may not be evident in parathyroidectomized rats during a normocalcaemic infusion of PTH. Another explanation for the discrepancy between findings *in vivo* and *in vitro* might be that the actions of PTH are mediated by a subfraction of adenylate cyclase activity (Catt & Dufau, 1977) not as accessible to direct inhibition by the analogue *in vivo* as *in vitro*. Since *in vitro* systems may in some way be altered or damaged in the procedure used for preparing membranes, the mineral ion transport responses to PTH *in vivo* may not be coupled to the same hormone-receptor-adenylate cyclase system as operates *in vitro*. Furthermore, intact cells maintained in monolayer culture may exhibit properties that are not representative of biological responses found *in vivo*. However, in such cases one might expect the *in vitro* systems to fail to detect weak, but definite, agonist like properties for the analogue. Some potent inhibitors of peptide hormone activity *in vitro* have been shown to behave as weak or partial agonists when evaluated *in vivo* (Rudinger, 1971; Poulsen, Burton & Haber, 1973; Burton, Poulsen & Haber, 1975; Vilchez-Martinez, Schally, Coy, Coy, Miller & Arimura, 1975; Ferland, Labrie, Savary, Beaulieu, Coy, Coy & Schally, 1976). Indeed, weak agonist properties of the PTH analogue have been demonstrated in the intact dog (Segre, Tully, Rosenblatt, Laugharn, Reit & Potts, 1979b). Since the analogue was not infused alone to parathyroidectomized rats, the possibility that it exhibited agonist activity in this system cannot be excluded. Indeed, a more recent study in thyroparathyroidectomized vitamin D-deficient rats maintained by calcium infusion has shown that the analogue has weak but definite agonist properties (e.g. phosphaturia and increased urinary cyclic AMP) when infused at molar concentrations 50 fold higher than that required to give a significant biological response to unsubstituted PTH-(1-34) (Horiuchi, Holick, Potts & Rosenblatt, unpublished observations).

Thus, the present data demonstrate an important dichotomy between *in vitro* and *in vivo* biological evaluation of PTH analogues and suggest that *in vitro* assays alone will not provide adequate screening for the design of potential antagonists. Our work emphasises the importance of a new direction in the evaluation of PTH analogues, namely, *in vivo* assays will need to be employed early rather than late in the assessment of biological properties of PTH.

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