HUMAN PARATHYROID HORMONE:
IMMUNOLOGICAL PROPERTIES OF THE
AMINO TERMINUS AND OF SYNTHETIC FRAGMENTS

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SUMMARY

1. Immunological assay systems specific to the amino-terminal region of parathyroid hormone were used to study the properties of human parathyroid hormone (HPTH 1-84) and of synthetic peptides. One of these peptides (HPTH 1-34A) had the sequence proposed by Niall, Sauer, Jacobs, Keutmann, Segre, O'Riordan, Aurbach & Potts (1974). The others (HPTH 1-34B and C, synthesized by different methods) had the sequence proposed by Brewer, Fairwell, Ronan, Sizemore & Arnaud (1972).

2. HPTH 1-84 and HPTH 1-34A behaved identically in a number of these systems. In some systems, especially those using antisera obtained by immunizing with HPTH 1-34A, differences were, however, found. These may be due to conformational or other physical differences or to the presence of an immunologically incomplete site in the fragment.

3. In systems in which HPTH 1-84 and HPTH 1-34A behaved identically the peptides with the sequence proposed by Brewer et al. (1972) were of low reactivity by 100-2000-fold.

Key words: amino terminus, parathyroid hormone, peptides.

Until recently, studies of the secretion of parathyroid hormone in man have been hindered because only a small quantity of human parathyroid hormone (HPTH) had been isolated, just enough to permit preliminary work on its immunological and chemical characterization (O'Riordan, Aurbach & Potts, 1969; O'Riordan, Potts & Aurbach, 1971a). However, it has now been isolated in sufficient quantity (Keutmann, Barling, Hendy, Segre, Niall, Aurbach, Potts & O'Riordan, 1974) to permit the elucidation of the sequence of the first thirty-seven amino acid residues (Niall, Sauer, Jacobs, Keutmann, Segre, O'Riordan, Aurbach & Potts, 1974). The human hormone was found to differ from the bovine hormone at three positions,

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and from the porcine hormone at two positions. A biologically active fragment comprising the first thirty-four amino acid residues of this sequence has been synthesized by the solid-phase method (Tregear, van Rietschoten, Greene, Niall, Keutmann, Parsons, O'Riordan & Potts, 1974).

Human parathyroid hormone has been isolated independently and the sequence of the first thirty-four amino acid residues determined by Brewer, Fairwell, Ronan, Sizemore & Arnaud (1972). Within the region 22-30, this sequence differs at three residues from that found by Niall et al. (1974). These changes, if correct, would be exclusive to the human hormone. Peptides having this sequence have been synthesized by Von Andreatta, Hartmann, Johl, Kamber, Maier, Riniker, Rittel & Sieber (1973) from Ciba-Geigy and by Beckman (Calif., U.S.A.) in 1973.

It is now therefore possible to examine the immunological properties of the amino-terminal region of HPTH, and studies of the immunoreactivity of these synthetic peptides may help to resolve the disagreements in the proposed sequences. Parathyroid hormone (PTH) contains several antigenic sites at different positions in the molecule (O'Riordan, Addison, Woodhead, Keutmann & Potts, 1971b) and antisera raised against the intact hormone are heterogeneous, containing antibody populations to different parts of the sequence. It is necessary therefore in examining the immunological behaviour of the amino-terminal region of PTH to use amino-terminal specific immunoassays. One way in which this specificity can be achieved is by blocking the carboxy-terminal reacting antibodies; the antiserum can be pre-saturated with a BPTH 53-84 fragment which has been prepared by enzymatic digestion of the intact molecule (Murray & Keutmann, 1973). Amino-terminal specificity can also be achieved by several other methods (Barling, Hendy, Evans & O'Riordan, 1974); for example, a synthetic amino-terminal fragment can be labelled and used as tracer in a standard immunoassay system. Alternatively, antibodies reacting with the amino-terminal regions of PTH can be extracted by use of an immunoadsorbent consisting of an amino-terminal fragment coupled to a solid phase. These antibodies can then be used either in a standard radioimmunoassay or labelled and then used in an immunoradiometric assay.

In this study we have compared the immunological properties of the available synthetic HPTH 1-34 peptides with those of the extracted human hormone, native bovine parathyroid hormone (BPTH) and a synthetic BPTH 1-34 fragment in a variety of immunoassay systems which were specific for the amino-terminal region of parathyroid hormone.

MATERIALS AND METHODS

Antisera

Antisera against bovine parathyroid hormone were raised by immunizing guinea-pigs with a crude extract of BPTH emulsified in complete Freund's adjuvant. Subcutaneous injections were given at monthly intervals. These antisera (AS 199 and 190) have been used extensively for the immunological characterization of parathyroid hormone (O'Riordan et al., 1971b).

An antiserum, G 127, was raised against the HPTH 1-34 peptide with the sequence proposed by Niall et al. (1974) by immunizing a goat. The peptide was emulsified in complete Freund's adjuvant and injected in multiple intradermal sites (Vaitukaitis, Robbins, Nieschlag & Ross, 1971).
Parathyroid hormone and hormone fragments

Bovine parathyroid hormone was prepared by the phenolic extraction method of Aurbach (1959) to yield a crude preparation, which was approximately 10% pure. This was subjected to gel filtration on Sephadex G-100 and ion-exchange chromatography on carboxymethyl-cellulose to yield a highly purified preparation which appeared homogeneous by polyacrylamide disc-gel electrophoresis and thin-layer chromatography.

The synthetic BPTH 1-34 fragment was obtained from Beckman, Palo Alto, California (lot no. 09012). It was stated to have a biological potency of 3100 MRC units/mg in the rat renal adenylate cyclase assay in vitro of Marcus & Aurbach (1969). The amount of peptide in this, and other preparations of fragments of parathyroid hormone, was established by amino acid analysis. BPTH 53-84 was prepared by limited tryptic digestion of highly purified BPTH after maleylation of the lysine residues (Niall, Keutmann, Sauer, Hogan, Dawson, Aurbach & Potts, 1970) and was again quantified by amino acid analysis.

The native human parathyroid hormone (HPTH 1-84) used in this study was a partially purified standard prepared by extracting parathyroid adenomas. This preparation (HT 67) has been carefully calibrated against highly purified HPTH by immunoassay, and was found to be 2.5% pure (O’Riordan et al., 1971a; Keutmann et al., 1974).

The synthetic HPTH 1-34 fragment with the sequence proposed by Niall et al. (1974) was synthesized by the solid-phase method and had a biological potency of 1050 MRC units/mg in the rat renal adenylate cyclase assay in vitro (Tregear et al., 1974). This peptide is designated in the text as HPTH 1-34A.

Synthetic fragments having the sequence proposed by Brewer et al. (1972) for the first thirty-four residues of HPTH were obtained from Beckman, Geneva, Switzerland (lot nos. 13013 and 14014). These had been prepared by solid-phase synthesis. No formal potency figure was assigned to these materials, but it was stated that in the rat kidney adenylate cyclase assay in vitro, half-maximal stimulation was obtained in the dose range 20-30 µg/ml (10% activity of BPTH 1-34 in the same assay system). This peptide is designated in the text as HPTH 1-34B.

Another peptide, having the sequence of Brewer et al. (1972), was synthesized by using fragment condensation techniques by von Andreatta et al. (1973) at Ciba-Geigy. No formal biological potency figure was assigned to this material, but it was stated to have hypercalcaemic activity in the thyroparathyroidectomized rat. This peptide is designated in the text as HPTH 1-34C.

Radioimmunoassay

BPTH 1-84, BPTH 1-34 and HPTH 1-34A were labelled with $^{125}$I to specific radioactivities of 200-300 mCi/mg by the chloramine-T method of Greenwood, Hunter & Glover (1963). The product was freed from damage fragments and changed iodine by adsorption on to microfine silica (Yalow & Berson, 1966).

Radioimmunoassay systems were modified to make them specific for the amino-terminal region of PTH (Barling et al., 1974). In some assay systems the antiserum used was presaturated with the BPTH 53-84 fragment so that only those antibodies raised against the amino terminus were reactive. In other assay systems amino-terminal specificity was conferred by using the $^{125}$I-labelled BPTH 1-34 fragment as tracer. In the assay with the anti-(HPTH 1-34A) antiserum, $^{125}$I-labelled HPTH 1-34A was employed as tracer.
Immunoradiometric assay

The immunoradiometric assay was performed as previously described (Addison, Hales, Woodhead & O’Riordan, 1971) but, again, modifications of the basic method were carried out so that the assays were specific for the amino-terminal region of the molecule (Barling et al., 1974). The amino-terminal reacting antibodies were selected for use in the assay by incubating the labelled antiserum with an immunoabsorbent consisting of the BPTH 1–34 peptide coupled to cellulose (BPTH 1–34 ImAd).

RESULTS

It is shown in Fig. 1 that in a radioimmunoassay using an anti-BPTH antiserum, AS 199, and 125I-labelled BPTH as tracer, HPTH 1–34A was not as effective in displacing the tracer as the native hormone. However, in this assay system the synthetic BPTH 1–34 peptide was likewise not as effective as the native bovine hormone. This is because of the presence of antibodies against other parts of the molecule, and in order to neutralize the carboxy-terminal reacting antibodies in this antiserum, it was pre-saturated with the BPTH 53–84 fragment.

When this had been done, as shown in Fig. 2, the native human hormone and the HPTH 1–34A fragment were equipotent in displacing the tracer. Both HPTH 1–34B and C peptides,
FIG. 2. Immunological properties of intact HPTH 1–84 (●) in comparison with the synthetic peptides HPTH 1–34A (○), 1–34B (■) and 1–34C (□), in an amino-terminus specific pre-saturation radioimmunoassay. Antiserum AS 199, pre-saturated with BPTH 53–84, was added at a final dilution of 1/100 000, and 125I-labelled BPTH 1–84 was used as tracer. Ratio B/F: see legend to Fig. 1.

FIG. 3. Comparison of the reactivity of intact HPTH 1–84 (●) with that of the synthetic peptide HPTH 1–34A (○), 1–34B (■) and 1–34C (□) in an amino-terminus specific labelled fragment radioimmunoassay. Antiserum AS 190 was used at a final dilution of 1/50 000, with 125I-labelled BPTH 1–34 as tracer. Ratio B/F: see legend to Fig. 1.
which have the sequence proposed by Brewer et al. (1972), were weakly reactive in this assay system so that $500 \times 10^{-13}$ mol of HPTH 1–34B or C were as reactive as $0.25 \times 10^{-13}$ mol of HPTH 1–34A or native human hormone.

In Fig. 3 are shown results with a different form of amino-terminal specific radioimmunoassay system using another anti-BPTH antiserum, AS 190, and $^{125}$I-labelled BPTH 1–34 as tracer: in this assay also the intact human hormone and the HPTH 1–34A peptide were equally effective in displacing the tracer. Again the two synthetic peptides having the sequence proposed by Brewer et al. (1972) reacted poorly in this system: $500 \times 10^{-13}$ mol of HPTH B or C gave the same displacement as $3.75 \times 10^{-13}$ mol of HPTH 1–34A or intact hormone.

![Fig. 4. Immunological properties of intact HPTH 1–84 (●) in comparison with the synthetic peptides HPTH 1–34A (○), 1–34B (■) and 1–34C (□), in an amino-terminus specific immunoradiometric assay. Amino-terminus reacting labelled antibodies from antiserum AS 199 were selected for use in the assay with an immunoadsorbent consisting of BPTH 1–34 linked to cellulose (BPTH 1–34 ImAd). The assay was separated with BPTH 1–84 ImAd.](image)

The intact human hormone and the HPTH 1–34A peptide also reacted very similarly in an immunoradiometric assay with labelled amino-terminal antibodies which had been selected from AS 199 (Fig. 4), although at high concentrations of added peptide the intact molecule was slightly more reactive than the 1–34 peptide. In this system the two peptides with the sequence of Brewer et al. (1972) were again only weakly reactive: 300 or $200 \times 10^{-15}$ mol of the HPTH 1–34B and C peptides respectively were required to be as effective as $3 \times 10^{-15}$ mol of HPTH 1–34A peptide in binding the labelled antibodies.

From Fig. 5 it is seen that the native human hormone and the HPTH 1–34A peptide did not react identically in every amino-terminal assay system. This figure shows results obtained in an immunoradiometric assay with amino-terminal labelled antibodies selected from AS 190.
Human parathyroid hormone

Fig. 5. Reactivity of (a) intact HPTH and HPTH 1-34A and (b) intact BPTH and BPTH 1-34 in an amino-terminus specific immunoradiometric assay. This assay was similar to that shown in Fig. 4, except that the labelled antibodies were prepared from antiserum AS 190.

Fig. 6. Comparison of the reactivity of intact HPTH 1-84 (●) and HPTH 1-34A (○) in (a) a radioimmunoassay and (b) an immunoradiometric assay using an antiserum raised against HPTH 1-34A. In the radioimmunoassay, the antiserum G.127 was used at a final dilution of 1/8000, with $^{125}$I-labelled HPTH 1-34A as tracer. In the immunoradiometric assay, the antibodies were selected for labelling with HPTH 1-34A ImAd, and the assay was separated with BPTH 1-34 ImAd. Ratio B/F: see legend to Fig. 1.
In this assay HPTH 1–34A peptide was less reactive (Fig. 5a) than the intact hormone. However, in the same immunoassay system, the bovine 1–34 fragment was likewise less effective in binding the antibody than the intact bovine hormone (Fig. 5b). Comparing the reactivity of HPTH 1–34A and BPTH 1–34 it is clear from this figure that there are immunological differences between the two synthetic amino-terminal peptides, which mirror differences seen in the native hormones.

In both radioimmunoassays and immunoradiometric assays using an antiserum to HPTH 1–34A (Figs. 6a and 6b) this peptide was found to be more reactive than the native human hormone. The corresponding bovine fragment (BPTH 1–34) was also found to differ from the intact bovine hormone in an equivalent manner in these assays.

**DISCUSSION**

Since there are a number of antigenic sites on parathyroid hormone and antisera against parathyroid hormone are heterogeneous, it is necessary to use region-specific assays when comparing the properties of a part of the molecule with those of the intact hormone. Some caution is necessary when such techniques are used, as a demonstration of immunological similarities between a synthetic fragment and the intact molecule cannot prove that a proposed sequence is correct because some errors in the structure might not affect immunological reactivity. On the other hand, when the fragment and the intact hormone seem to differ immunologically it may not mean that the proposed amino acid sequence is incorrect because immunological differences could be produced in other ways. For example, an antigenic site in a fragment might be incomplete, because of the absence of residues further along the sequence of the intact hormone. Alternatively the presence of a free carboxyl group at the end of the fragment might affect the reactivity of neighbouring antigenic sites through charge differences. Immunological differences would also be produced if the steric configuration of the intact hormone and the fragment were to differ. Reasons such as these may account for immunological differences which have been found between BPTH 1–34 and the same region of intact bovine parathyroid hormone (Barling et al., 1974).

It has been known for some time that bovine, porcine and human parathyroid hormones differ immunologically. Now that the complete sequences of bovine and porcine parathyroid hormones are known and there is information on the structure of part of human parathyroid hormone, it is possible to investigate the structural basis for these immunological differences. The sequence of the amino-terminal region of human parathyroid hormone reported by Niall et al. differs from that of BPTH at three positions: at position 1 human parathyroid hormone contains serine instead of alanine, at position 7 leucine instead of phenylalanine, and at position 16 asparagine in place of serine. It is shown here that, in one system at least (Fig. 5), the N-terminal reactivity of BPTH and HPTH differ, and so some of the immunological differences (that have hampered development of radioimmunoassays for HPTH) must stem from changes in sequence at the amino terminus.

When the properties of intact human parathyroid hormone were compared with those of the synthetic peptide prepared with the sequence proposed by Niall et al. (1974), immunological similarities were found in a number of region-specific assay systems. In some systems, however, the behaviour of HPTH 1–34A differed from that of HPTH 1–84, though when these immunological differences were found, it was in systems in which BPTH 1–34 was also
found to differ immunologically from BPTH 1–84. The apparent immunological differences were greatest in assays with an antiserum to the synthetic fragment HPTH 1–34A. These were highly sensitive for the fragment, although the intact hormone had lower reactivity, by a factor of approximately 10. Clearly, HPTH 1–34A differs as an immunogen from the equivalent sequence in the intact hormone. Such differences would be expected if antibodies in this antiserum are predominantly directed towards the carboxy-terminal part of the fragment, which as a result of the presence of a free carboxyl group on the terminal residue 34, differs in charge from the intact hormone at this point.

The amino-terminal specific antibodies (from AS 199 and AS 190) used here react especially well with an antigenic site in the region 22–34 of parathyroid hormone—we have shown this with a series of synthetic fragments of the amino-terminal region of BPTH. These antibodies therefore react with the region in human parathyroid hormone in which the structure proposed by Brewer et al. (1972) differs from that proposed by Niall et al. (1974). In the sequence reported by Brewer et al. (1972) there is a glutamine residue (rather than glutamic acid) at position 22, a lysine (rather than leucine) at position 28 and a leucine (rather than aspartic acid) at position 30. These changes would give the sequence a net charge difference of three in this region and might be expected to give immunological differences. It is shown here that the synthetic peptides made with the sequence proposed by Brewer et al. (1972) have low affinity for the antibodies: 100- to 2000-fold greater amounts of them than of HPTH 1–34A are required to give the same effect. This lack of reactivity was seen in amino-terminal reacting systems in which HPTH 1–34A and intact HPTH reacted identically. Since the only differences between the synthetic peptides HPTH 1–34A, B and C span the region 22–30, these systems must have been sensitive to an immunologically active site in this region of the hormone. As expected therefore the charge differences have produced immunological differences. The lack of reactivity was seen in both batches of HPTH 1–34B, prepared by solid-phase synthesis, and in HPTH 1–34C prepared by an entirely different method, and is unlikely therefore to be due to defects in the peptide syntheses.

For reasons given already, the fact that intact human parathyroid hormone and the peptide with the sequence proposed by Niall et al. (1974) behaved similarly cannot be used to prove definitively that this sequence is correct. However, since the peptides with the sequence proposed by Brewer et al. (1972) behaved very differently from human parathyroid hormone in these systems, it would seem that caution is necessary when these peptides are used for studies of parathyroid hormone in man.

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