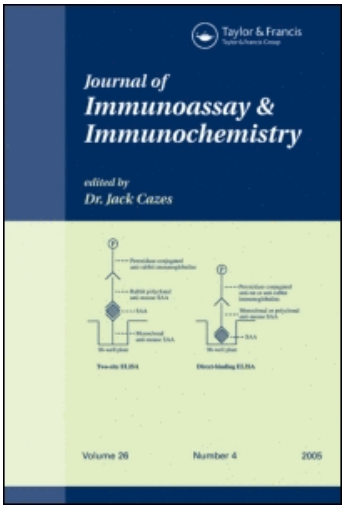

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

An Effect of Parathyroid Hormone on the Epiphyseal Plate and Osteoblasts: Studies Towards a Cytochemical Bioassay

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To cite this Article Dunham, Jane and Chayen, J.(1983) 'An Effect of Parathyroid Hormone on the Epiphyseal Plate and Osteoblasts: Studies Towards a Cytochemical Bioassay', Journal of Immunoassay and Immunochemistry, 4: 4, 329 — 338
To link to this Article: DOI: 10.1080/15321818308057013
URL: http://dx.doi.org/10.1080/15321818308057013

JOURNAL OF IMMUNOASSAY, 4(4), 329-338 (1983)

AN EFFECT OF PARATHYROID HORMONE ON THE EPIPHYSEAL PLATE AND OSTEOBLASTS: STUDIES TOWARDS A CYTOCHEMICAL BIOASSAY.

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ABSTRACT

There are reports of an apparent dissociation between the responses of kidney and bone to parathyroid hormone (PTH): certain fragments or analogues have been shown to have differential activity in these two tissues. Consequently, cytochemical bioassay techniques have been applied to the development of an assay for PTH using the epiphyseal plate of the rat metatarsal as the target organ. Glucose 6-phosphate dehydrogenase activity in the zone of hypertrophic cartilage is stimulated in a log-linear fashion with increasing concentrations of parathyroid hormone over the range 0.001 - 1.0 pg/ml. A parallel response was found in the osteoblasts of the metaphysis. Measurements of a normal and a hyperparathyroid plasma sample showed good parallelism to the standard graph and good discrimination. Addition of a PTH - specific antibody inhibited both the effect of the standard preparation of the hormone, and the response induced by dilutions of a normal plasma.

(KEY WORDS: Parathyroid hormone, Cytochemical bioassay, Epiphyseal plate, Glucose 6-phosphate dehydrogenase).

INTRODUCTION

There is both clinical and experimental evidence to suggest an apparent dissociation between the responses of kidney and bone to parathyroid hormone and differential activities of certain fragments or analogues on these tissues have been demonstrated. Thus Martin et al (1) showed the bovine 1 - 34 fragment to be active but the intact hormone to be inactive in stimu-

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lating the production of cyclic AMP in isolated perfused bone. In this system Nle^{8} Nle^{18} Tyr^{34} bPTH(3-34) amide was an agonist whereas it was an antagonist in all kidney bioassay systems. In the rat renal membrane assay bPTH(2-24) has been shown to have only 2% of the activity of the intact hormone whereas it had 65% of the calcium-mobilizing activity from chick bone (2). A clinical example is seen in some cases of pseudohypoparathyroidism where skeletal responsiveness, albeit variable, and renal resistance (3) suggest that the circulating form of the hormone may be active on the one and not on the other. The cytochemical bioassay for PTH (4), using guinea pig kidney as the target organ, is now well established. Potency estimates have shown that the structure/function requirements of this assay resemble those of the rat renal adenylate cyclase assay rather than the in vivo chick hypercalcaemia assay (5). This finding emphasized the need for a cytochemical bioassay for the hormone based on bone.

METHOD

Female Wistar rats (50 - 100g) were killed by asphyxiation with nitrogen. This is a within-animal assay with one rat used for each assay. The metatarsals were removed and cut in half to isolate the distal portion containing the epiphyseal plate. Each segment was placed on a defatted lens tissue on a metal grid in a vitreosil dish. Each dish contained Trowell'sT8 medium, pH 7.6, added until the level of the metal grid was reached. The dishes were then placed in individual culture chambers and maintained at 37°C in an atmosphere of 95% O_2: 5% Co_2 for 4 - 4½ hours. After this period the T8 medium was removed and replaced with fresh T8 - medium containing a dilution either of the standard preparation of the hormone or of the test plasma. The standard reference preparation used in this study was NIBSC code 77/533 bovine parathyroid hormone. Dilutions were usually from 0.001 pg/ml to 1.0 pg/ml, with plasma dilutions from

1 : 50 to 1 : 10,000. 0.1% hypoparathyroid plasma was added to the 1.0 pg/ml standard dilution and diluted accordingly.

After exposure to hormone for eight minutes the segments were transferred to a 5% solution of polyvinyl alcohol for 5 sec. This provides a protective coating for the epiphyseal plate so enabling easier sectioning of the segment and better adhesion to the microtome chuck. The segments were then chilled in n-hexane (boiling range 67°C to 70°C; 'low in aromatic hydrocarbons' grade), maintained at - 70°C by a surrounding bath of crushed CO_2 - ice and industrial spirit. After 25 - 30 sec the segments were placed in pre-cooled cold tubes and maintained at -70°C. These techniques have been fully discussed by Chayen et al (6). The segments of metatarsals were mounted on microtome chucks, covered by a layer of a 5% solution of polyvinyl alcohol to give better adhesion. They were sectioned at 10 µm thickness on a Jung 1130 rotary microtome. The knife used was a tungsten-tipped knife (Autoradiographic Products Ltd. Cheshire). This microtome was contained in a Bright's cryostat at -25°C to - 30°C. Solid carbon dioxide was placed around the haft of the knife. The sections were stored in the cryostat whilst the reaction-medium was prepared.

The reaction-medium contained glucose 6-phosphate disodium salt, 5mM; NADP, 3mM in 0.05M glycyl glycine buffer containing 30% polyvinyl alcohol (grade G04/140, Wacker Chemicals Ltd., Walton-on-Thames, Surrey, U.K.), with 0.3% chloroform-purified neotetrazolium chloride (Serva). The intermediate hydrogen-acceptor, phenazine methosulphate (3mM), was added just before use. Unless otherwise stated, all the chemicals were obtained from Sigma.

The final pH of the reaction-medium was pH 8.0. The medium was saturated with nitrogen since oxygen competes with neotetrazolium chloride for reducing equivalents. The reaction medium was poured into Perspex rings placed around the sections which had been warmed to 37°C and the reaction was done for 45 min at 37°C. The sections were then washed in distilled water and mounted in Farrants' medium shortly before the reaction-product was to be measured.

The glucose 6-phosphate dehydrogenase activity was measured by means of a Vickers M85 scanning and integrating microdensitometer (x 40 objective; spot diameter 0.5μm in the plane of the section; and at a wavelength of 585nm). The mask encompassed one cell and the results were expressed in absolute units of extinction (mean integrated extinction x 100) per cell.

The cells selected for measurement were the cells in the hypertrophic zone of the epiphyseal plate.

RESULTS

A high level of glucose 6-phosphate dehydrogenase activity was measured in the cells of the epiphyseal plate taken directly after the animal was killed (DC in Fig. 1). After the culture period followed by exposure to Trowell's T8-medium alone, the enzyme activity was much reduced (C + T8; Fig.1). Segments of the tissue exposed to 0.1pg/ml of parathyroid hormone for various times showed maximal glucose 6-phosphate dehydrogenase activity after 8 minutes exposure to the hormone. This activity was stimulated in a log-linear fashion with increasing concentrations of parathyroid hormone over the range 0.001 - 1.0pg/ml (Fig. 1).

The cells selected for measurement were those of the zone of hypertrophic cartilage. The cells in the proliferating zone showed a similar response but were of a less suitable size to fill the mask when measuring the enzyme activity. The osteoblasts of the metaphyseal trabeculae were measured and showed a parallel response to that of the cartilage cells of the hypertrophic zone, although the absolute level of enzyme activity of the former was somewhat greater (Fig. 2). The variability within the osteoblastic layer however, meant these cells were not ideal for measurement.

Addition of an antibody specific for PTH (1:250,000 Burroughs Wellcome AS211/32) to a standard preparation of the hormone annulled the effect of the hormone. In the presence of the antibody, the enzyme activity in the segments treated with dilutions of 1.0 pg/ml and 0.1 pg/ml was reduced to

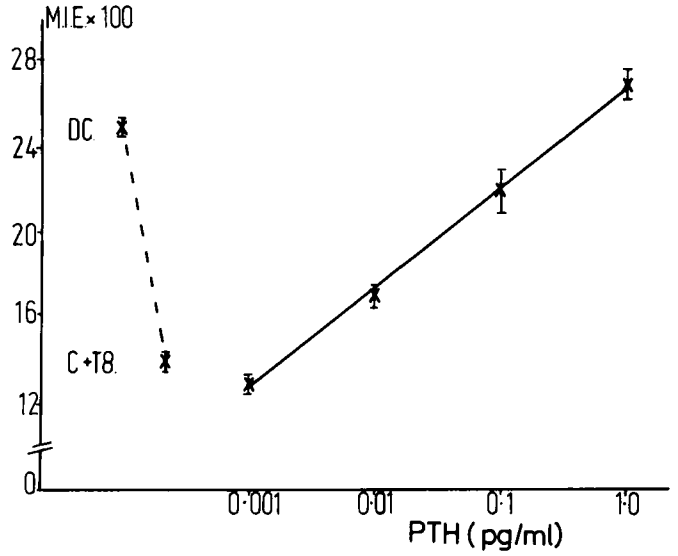

Fig. 1. The response of glucose 6-phosphate dehydrogenase activity (MIE x 100) to different concentrations of a standard preparation of PTH after 8 min exposure to the hormone. The activities measured in a specimen that had not been maintained in vitro (DC) and in one after maintenance culture without exposure to the hormone (C + T8) are included for comparison.

that found at the 0.01 and 0.001 pg/ml levels in the control preparations; that is the antibody caused 99% inhibition.

Dilutions of a normal plasma (1 : 50 and 1 : 500) were assayed against the standard preparation of the hormone. The two dilutions gave a response that was parallel to the standard graph and assayed at 2.4 pg/ml. The same plasma was assayed using the guinea pig kidney as the target-organ. The plasma again gave a response that was parallel to the standard graph and assayed at 1.9 pg/ml (Fig. 3).

Plasma taken from a normal subject and from a subject with primary hyperparathyroidism just prior to undergoing a parathyroidectomy were assayed

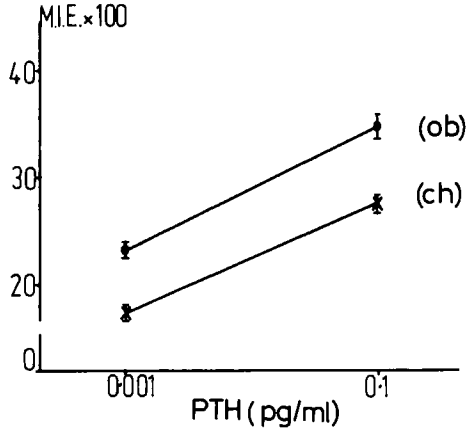

Fig. 2. The activities of glucose 6-phosphate dehydrogenase (MlE x 100) in osteoblasts (ob) and in chondrocytes of the hypertrophic zone (ch) under the influence of two concentrations of PTH, acting for 8 min. The responses are clearly similar.

at dilutions of 1 : 100 and 1 : 1000 and at 1 : 1000 and 1 : 10,000 respectively. The assay discriminated well between the two plasmas with values of 0.5pg/ml and 240pg/ml (Fig.4).

Addition of the antibody B.W. AS211/32 to dilutions of a normal plasma annulled the parallelism of response. Dilutions of the control plasma (1 : 100 and 1 : 1000) gave results of 26 and 16 pg/ml respectively, whilst in those containing the antibody the response was very low and completely non-parallel (Fig. 5). The 1 : 50 dilution assayed at 0.1 pg/ml; the 1 : 500 dilution was too low to be assayed and gave the anomalous result expected at dilutions outside the range of the assay. Addition of exogenous PTH to a normal plasma resulted in 95% recovery of the hormone.

Index of Precision.

The mean index of precision from 6 assays selected at random was 0.16 ± 0.05 (mean ± SEM).

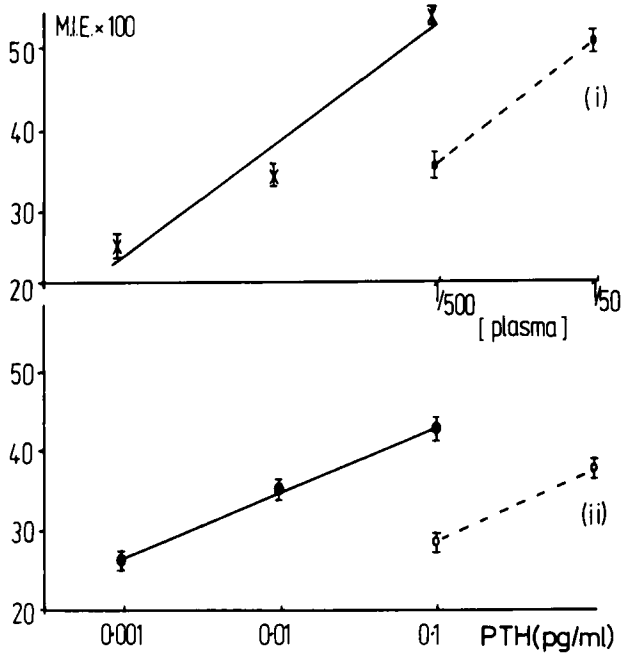

Fig. 3. Two assays of the same sample of plasma, (i) with the epiphyseal plate as the target-organ and (ii) with the guinea-pig kidney as the target-organ. The broken line links the results obtained with the dilutions of the plasma; the solid line represents the dose-response to the standard reference preparation of PTH.

DISCUSSION

Earlier studies (7) on the activities in this system of alkaline phosphatase, lactic, succinate and glucose 6-phosphate dehydrogenases, under the influence of PTH, indicated that the effect of the hormone on the last gave the best discrimination. It has now been shown that glucose 6-phosphate dehydrogenase activity in the epiphyseal plate is stimulated in a log-linear fashion with exposure to increasing concentrations of PTH. This effect was annulled by the addition of a specific antibody.

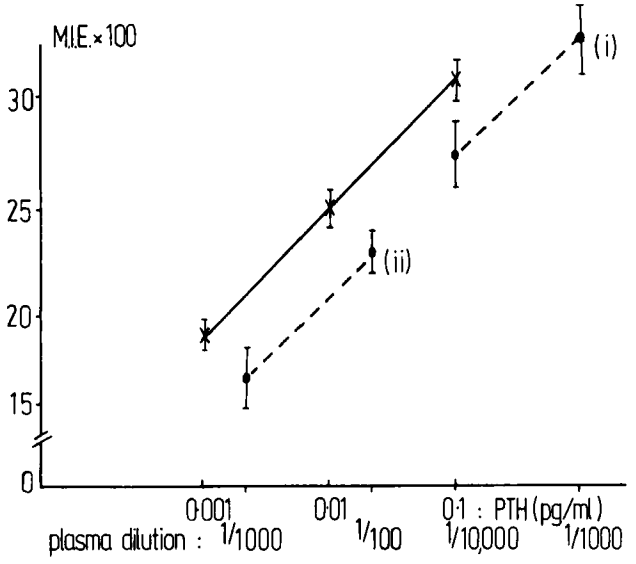

Fig. 4. The assay of plasma from (i) a hyperparathyroid and (ii) a normal subject measured against the same standard dose-response (crosses and solid line).

Serial dilutions of plasma gave a response that was parallel to that produced by a standard preparation of the hormone and which was obliterated by the addition of the antibody. It is therefore reasonable to conclude that the response measured in this system represented PTH or PTH-like activity.

Although the cells selected for measurement were the cells of the hypertrophic zone of the epiphyseal plate, the osteoblasts lining the trabeculae of the metaphysis were equally responsive to parathyroid hormone. These cells showed a response that was parallel to that found in the cartilage cells, although the absolute level of enzyme activity was greater in the former. Thus the only criterion for selecting the cartilage cells was the ease of measurement. The levels of parathyroid

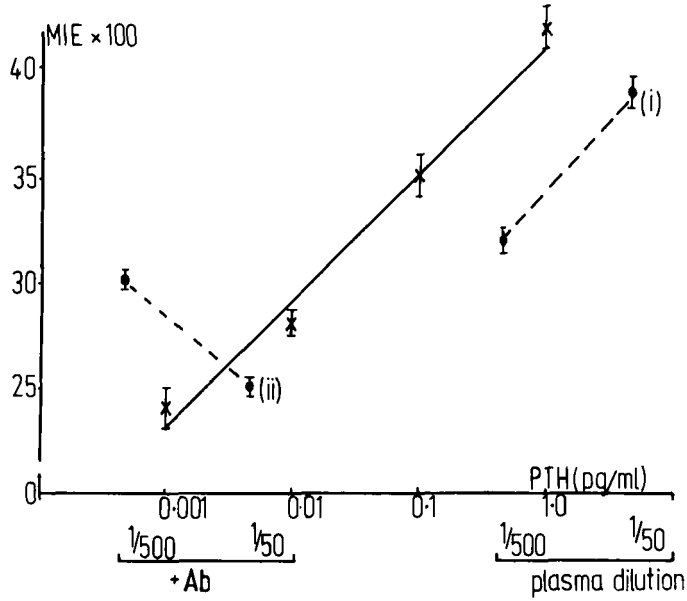

Fig. 5. The assay of a normal plasma (i) and the effect of pre-incubation of the same plasma with a specific antibody (ii). The standard dose-response graph is shown with crosses, and a solid line.

hormone found in the plasma samples measured so far have not varied greatly from the levels found using the kidney assay. The normal values lie within the range already established for the cytochemical bioassay and the hyperparathyroid result was of the order expected from this class of patient.

There is much yet to be done before the results, described in this communication, constitute a validated bioassay. However they do indicate that chondrocytes and osteoblasts of the epiphyseal plate of rat metatarsal bones respond to low concentrations of PTH, in a log-dose fashion, that may form a basis for a bioassay of this hormone acting on bone. The interest of these findings will lie in situations where the cytochemical renal bioassay does not fully explain the clinical

findings so that it may be pertinent to determine whether, or not, the sample stimulates these bone-related cells.

ACKNOWLEDGEMENT

We are grateful to the Arthritis and Rheumatism Council for Research for support for this work. We are indebted to the National Institute for Biological Standards and Control for the preparations of bovine PTH.

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