Parathyroid hormone bioassay using human kidney cortical cells in primary culture

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Abstract. The biological activity of parathyroid hormone (PTH) has been investigated by measuring intracellular accumulation of cyclic AMP (cAMP) in human kidney cortical cultures. Enzyme dispersed cortical cells from non-invaded kidney poles of patients undergoing nephrectomy for cancer were used after 5 days of primary culture. Bovine PTH (1–84) produced a significant increase of cAMP accumulation in cultured cells at a dose (53.7 ng/ml) 10-fold lower than that found for the minimal stimulatory effect when using preparations of human purified plasma membranes. The action of bovine PTH (1–84) was very rapid, a response was detected after 5 min and a ceiling effect after 30 min. Cortical cells showed a slightly lower sensitivity to synthetic bovine PTH (1–34) (half maximal increase dose: 0.66 μg/ml), compared to bovine PTH (1–84) (half maximal increase dose: 0.32 μg/ml), but revealed a higher sensitivity to human PTH purified from the medium of parathyroid cell cultures (half maximal increase dose: 11.2 ng/ml). Arginine-vasopressin (AVP) also increased the cAMP accumulation of kidney cortical cultured cells, with a potency and efficacy lower than that of human 'culture' PTH, while in kidney medullary cells in primary culture AVP exerted a strong response and the effect of PTH was poor or absent. Calcitonin and glucagon were weak stimulators of kidney cortical cell cAMP accumulation.

Different methods have been proposed to test the biological activity of parathyroid hormone (PTH). In vivo assays are almost always based on the calcium-mobilizing effect of PTH in parathyroidectomized rats (Munson 1955) or in chicks (Parsons et al. 1973). In vitro assays may be based on the release of calcium from mouse calvaria into tissue culture medium (Zanelli et al. 1969), or on the stimulation of glucose-6-phosphate dehydrogenase activity of the kidney distal convoluted tubules evaluated by a cytochemical method (Chambers et al. 1978). Most bioassays have revealed a PTH effect on the adenylate cyclase-cyclic AMP system in target organs from different animal species (Goltzman 1979). However these methods appear to be active at a supraphysiological PTH concentration (Parsons 1979).

The present report investigates whether human kidney cortical cells in primary culture may be used for a specific and sensitive bioassay of PTH by measuring intracellular accumulation of cyclic AMP (cAMP). The effects of other peptide hormones active on the kidney, such as arginine-vasopressin (AVP), calcitonin (CT) and glucagon, have also been studied; cultures derived from the renal medulla were used as control.

Materials and Methods

Renal tissue was obtained from non-invaded kidney poles of patients undergoing nephrectomy for cancer; no treatment was given during the week prior to surgery. The normal histological identity of kidney tissue was confirmed by conventional histochemical staining and by observing frozen sections. Each experiment utilized cells derived from a single kidney.

Kidney cell dispersion and culture

The technique used was from the original method described for thyroid tissue (Toccafondi et al. 1980). After excision and collection all handling was performed under aseptic conditions. Within 30 min of removal, the renal
cortex and medulla were divided with scissors, the adhering connective tissue was also removed and the remainder minced by a scalpel into small fragments. Tissue fragments, washed twice in phosphate buffered saline (PBS), were suspended in Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, containing glucose 1.0 g/l, bovine serum albumin (BSA) 3.0 g/l, deoxyribonuclease I 0.05 g/l and supplemented with trypsin 2.5 g/l. Trypsinization was carried out for 10 min at 37°C in a 95% O₂/5% CO₂ atmosphere. Trypsinized kidney fragments were mechanically dispersed by pipetting with a fine siliconized pipette after washing 3 times with KRB, centrifuged each time at 500 x g and re-washed 3 times with KRB free of Ca²⁺ and Mg²⁺. Freshly isolated kidney cells were suspended in McCoy’s 5a medium with sodium bicarbonate 2.2 g/l supplemented with 20% foetal calf serum (FCS) and seeded at 1 x 10⁵ cell concentration per cm² in each well of 2 cm²—Falcon multiwell plates. At the end of dispersion cell viability was about 95% or more, assessed by trypan blue exclusion. The culture medium was changed on the second and fourth day of culture; if culture was prolonged the medium was changed every 3 days.

**Stimulation of adenylate cyclase-cAMP system**

After 5 days of culture the medium was removed and replaced with 0.2 ml of KRB buffer containing 0.6 mM 3-isobutyl-1-methyl-xanthine (IMX), glucose (10 mM) and BSA (2.0 g/l); KRB buffer was equilibrated at 37°C before adding to the cells. Stimuli were added in 20 μl of PBS alone. Incubations were carried out at 37°C in air with 10% CO₂ and ended by adding cold absolute ethanol (0.2 ml).

**Plasma membrane preparation and adenylate cyclase activity assay**

Normal human kidney cortical plasma membranes were purified by differential centrifugation according to Toccafondi et al. (1979), with minor modifications, adding 10% (v/v) dimethylsulphide to the homogenizing and Tris/HCl buffers. Purified plasma membranes stored in liquid nitrogen were used within 7 days. Adenylate cyclase activity was determined according to Albano et al. (1973), evaluating the cAMP formed from unlabelled ATP (2 mM) in the presence of an ATP regeneration system (Toccafondi et al. 1979).

**Cyclic AMP and DNA measurement**

Cyclic AMP accumulation was measured in the freeze-dried 2000 x g supernatant of the ethanol treated homogenate by a competitive protein-binding assay (Brown et al. 1971). With this method it was possible to measure the cAMP concentration from a dose of 0.25 pmol cAMP/tube (i.e., 5 pmol cAMP/ml). The binding protein showed a cross-reactivity with cyclic GMP and other nucleotides which was always less than 4% in the different batches.

The intra-assay error never more than 4.8%, while the inter-assay never exceeded 7.3%.

In preliminary experiments there was little change in intracellular cAMP between 5 and 60 min of incubation after 5 days of culture. At the same time extracellular cAMP was constantly 10–15% of the intracellular concentration and the same proportion was maintained under stimuli. Total cAMP (cell plus medium) was therefore a reasonable approximation of intracellular cAMP.

A trace amount of [3H]cAMP (0.8 kBq) was added to control incubation wells and the percentage of recovery was calculated. Some 2–4% of the labelled cAMP was adherent to plastic culture wells and another 6–8% remained in the cellular pellet. The amount of non-hydrolyzed cAMP present in the supernatant was calcu-

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**Fig. 1.**

A: Gel filtration on Biogel P10 (2.5 × 60 cm column) of culture medium (2 ml) of parathyroid cells in primary culture. Human PTH concentration was determined in each fraction by radioimmunoassay using an anti (1—84) PTH serum and expressed as μg equivalent of NIBSC 75/549 International Standard. Arrows indicate the elution volume of Blue Dextran (V₀), of 125I-labelled bovine PTH (1—84) and of potassium chromate (Vₙ): B: Gel filtration on the same column of fractions Nos. 34–36, concentrated by ultrafiltration on a UM 2 Diaflo membrane, of the previous gel filtration.
Table 1.
Cellular growth and responsiveness of adenylate cyclase system to human 'culture' PTH (14 ng/ml) in human kidney cells during the first decade of primary culture.

<table>
<thead>
<tr>
<th>Days of culture</th>
<th>DNA content**</th>
<th>cAMP*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>hPTH</td>
</tr>
<tr>
<td>2</td>
<td>0.63 ± 0.04</td>
<td>0.20 ± 0.06</td>
<td>1.92 ± 0.18</td>
</tr>
<tr>
<td>5</td>
<td>0.90 ± 0.06</td>
<td>0.24 ± 0.01</td>
<td>4.11 ± 0.27</td>
</tr>
<tr>
<td>7</td>
<td>1.08 ± 0.13</td>
<td>0.35 ± 0.04</td>
<td>5.07 ± 0.50</td>
</tr>
<tr>
<td>10</td>
<td>1.26 ± 0.15</td>
<td>0.47 ± 0.06</td>
<td>4.55 ± 0.22</td>
</tr>
</tbody>
</table>

* Expressed as pmol/μg DNA (mean ± SD). ** μg per dish (16 mm diameter).

lated by a difference in binding to protein with respect to an untreated equal amount of [3H]cAMP. The recovery of non-hydrolyzed [3H]cAMP in the supernatant was maximal (90%) in the presence of 0.5 mM IMX (final concentration); using lower amounts of IMX the recovery was proportionally decreased.

Differences in cellularity were corrected for the DNA content of the pellet (Kissane & Robins 1958) and results expressed as pmol/μg DNA. cAMP measurements were performed in duplicate. All experiments were performed in quadruplicate and repeated 3 times. Statistical analysis was performed by means of one-way analysis of variance.

** Purification of human parathyroid hormone from cultured human parathyroid cells**

Parathyroid cell cultures from enzyme dispersed parathyroid cells were taken from surgically removed human hyperplastic parathyroid tissue. Cultures were carried out as for kidney cells, with low calcium concentrations (0.9 mM) in the medium. Culture medium (2 ml) was purified on a Bio Gel P10 column (2.5 x 60 cm) in ammonium acetate, pH 4.5, containing 1% BSA, and PTH content was monitored in each fraction by radioimmunoassay using an anti-(1-84) PTH serum (see below) (Fig. 1A). Fractions nos. 34–36 were concentrated.

![Figure 2](image)

**Fig. 2.**
Time-course of cAMP accumulation in human kidney cortical cultured cells produced by 0.43 μg/ml bovine PTH (1–84) (●), 0.1 μg/ml synthetic bovine PTH (1–34) (○) and 7.0 ng/ml human 'culture' PTH (♦). Open points (○) represent baseline cAMP levels. Results are the mean ± sd of the cAMP response at each time.
by ultrafiltration on a Diaflo membrane (UM 2) and again purified by gel filtration on the same column (Fig. 1B); fraction no. 35 of this second gel filtration, divided in 0.2 ml aliquots, was stored at −20°C until use.

Human 'culture' PTH was indistinguishable from purified human PTH (NIBSC 75/549) in a range of different immunoassay systems. The concentration of human 'culture' PTH was measured at serial dilution by radioimmunoassay (Hehrmann 1980) using an anti-
(1–84) PTH serum (S-478) and expressed as nanogram equivalent of NIBSC International Standard 75/549.

Materials
Human PTH (1–84) (NIBSC 75/549), bovine PTH (1–84) (NIBSC 77/533) and human CT (NIBSC 70/234) were a gift from Dr. J. M. Zanelli, National Institute for Biological Standard and Control, Holly Hill, London, UK. The amino terminal synthetic fragment of the bovine PTH (1–34) was purchased from Beckman BioProducts, Geneva, Switzerland; salmon CT from Sandoz, Basel, Switzerland; Desaminocys1-D-arginine8-vasopressin from Ferring, Malmö, Sweden; porcine glucagon from Novo Industri, Copenhagen, Denmark. McCoy's 5a medium and FCS were purchased from Gibco, Grand Island, New York, USA; adenosine-3',5'-monophosphoric acid, IMX from Sigma, St. Louis, USA; deoxyribonuclease I from Boehringer Biochemia, Mannheim, W. Germany; [2,8-3H]adenosine-3',5'-cyclic-phosphate was purchased from the Radiochemical Centre, Amersham, UK; Bio Gel P10 from BioRad, Richmond, Calif., USA; multiwell plates from Falcon, Oxnard, Calif., USA; Diaflo ultrafiltration membranes from Amicon Co., Lexington, Mass., USA; trypsin and all reagents of analytical grade from Merck, Darmstadt, W. Germany.

Results

Characteristics and behaviour of kidney cortical cells during the first decade of culture
On the fifth day of culture the cells, when observed by phase-contrast microscopy, showed an epithelial appearance with round or polygonal cells, with a single large prominent nucleus, in 92–94% of cases, while the remaining cells were spindle-shaped with scarce cytoplasm. On the tenth day of culture the percentage of fusiform cells increased to almost 18–20%, while epithelial cells showed a large, flat cytoplasm. Culture therefore appeared to be almost confluent.

The modification of basal and post-PTH stimulated cAMP accumulation in human kidney cortical cell cultures was also investigated for 10 days (Table 1). Basal cAMP accumulation and DNA content of each well increased progressively and almost doubled between the second and tenth days. Even though the percentage increase of cAMP accumulation above basal values in response to human 'culture' PTH was maximal after 5 days of culture, it declined progressively thereafter.

Fig. 3.
Dose-response curves of cAMP accumulation in human kidney cortical cultured cells produced by different amounts of bovine PTH (1–84), synthetic bovine PTH (1–34) and human 'culture' PTH. The shaded area represents two standard deviations of mean baseline cAMP levels.
Table 2.
Effect of human serum on cAMP accumulation in human kidney cortical cells at baseline and after stimulation with human 'culture' PTH (hPTH). Cultured cells were incubated with different amounts of hPTH for 30 min in absence or presence of 50% normal human serum.

<table>
<thead>
<tr>
<th>hPTH (ng/ml)</th>
<th>cAMP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No serum</td>
</tr>
<tr>
<td>0</td>
<td>0.75 ± 0.25</td>
</tr>
<tr>
<td>3.5</td>
<td>3.71 ± 0.81</td>
</tr>
<tr>
<td>7.0</td>
<td>6.51 ± 0.94</td>
</tr>
<tr>
<td>14.0</td>
<td>9.89 ± 1.10</td>
</tr>
</tbody>
</table>

* Expressed as pmol/μg DNA (mean ± sd).

**PTH dose-response characteristics (Fig. 3)**
When kidney cortical cells were incubated for 30 min with a wide range (0.6 ng/ml to 4.2 μg/ml) of human 'culture' PTH, bovine PTH (1–84) (NIBSC 77/533) and synthetic bovine PTH (1–34), a significant increase of cAMP accumulation was detected at 1.7 ng/ml for human 'culture' PTH, at 53.7 ng/ml for bovine PTH (1–84) and at 100 ng/ml for bovine synthetic PTH (1–34) (P < 0.01 in all cases). The rate of increase was dose-dependent up to a concentration of 28 ng/ml for human 'culture' PTH, of 1.7 μg/ml for bovine PTH (1–84) and of 4.2 μg/ml for synthetic bovine PTH (1–34). The half-maximal dose, calculated on the dose-response curves, was 11.3 ng/ml for human 'culture' PTH, 0.32 μg/ml for bovine PTH (1–84) and 0.66 μg/ml for synthetic bovine PTH (1–34).

The effect of normal human serum on cAMP response to human culture PTH was also investigated with different amounts of human PTH for 30 min in the absence or presence of 50% pooled serum from normal healthy subjects. As shown in Table 2 no significant effect of serum was detectable either on basal or post-PTH stimulated cAMP accumulation.

cAMP response to other peptide hormones active at the kidney level
The effect of several peptide hormones, active at the kidney level through the adenylate cyclase-cAMP system, was also studied. Table 3 shows the effect of maximal doses of human CT, salmon CT, glucagon and AVP on kidney cortical cell cAMP content. Lower doses of human CT and glucagon were no longer active in increasing cAMP levels, while serial dilutions of salmon CT and AVP appeared also to be active at lower doses (Fig. 4). In fact the minimal effective dose of salmon CT was 1 x 10^-8 M while that of AVP was 1 x 10^-9 M.

Adenylate cyclase activity of human kidney cortical plasma membranes
As shown in Table 4 the basal adenylate cyclase activity of human kidney cortical plasma membranes was 10.2 ± 0.4 pmol cAMP/10 min/mg of protein (mean ± sd). Bovine PTH (1–84) (NIBSC 77/533) produced a dose-dependent increase of the renal adenylate cyclase activity from a concentration of 0.4 μg/ml reaching a maximal effect at a dose of 3.4 μg/ml.

Table 3.
Effect of different peptide hormones on human kidney cortical cells after 5 days of primary culture. Cyclic AMP accumulation was evaluated in presence of 0.6 mM 3-isobutyl-1-methyl-xanthine, after 30 min incubation at 37°C and expressed as pmol/μg DNA (mean ± sd).

<table>
<thead>
<tr>
<th></th>
<th>Concentration (M)</th>
<th>cAMP (pmol/μg DNA)</th>
<th>Per cent increase above basal levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>0.55 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>Human calcitonin</td>
<td>10^-6</td>
<td>1.04 ± 0.09</td>
<td>89**</td>
</tr>
<tr>
<td>Salmon calcitonin</td>
<td>10^-6</td>
<td>1.62 ± 0.18</td>
<td>198**</td>
</tr>
<tr>
<td>Glucagon</td>
<td>10^-6</td>
<td>0.86 ± 0.04</td>
<td>57*</td>
</tr>
<tr>
<td>Arginine-vasopressin</td>
<td>10^-6</td>
<td>6.27 ± 0.72</td>
<td>1040***</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001.
Effect of AVP and PTH in kidney medullary cultured cells

In order to verify the specificity of action of hormones tested at cortical level and the validity of the present method we also studied the effect of AVP and human 'culture' PTH on cAMP levels in cultured human renal medullary cells.

The mean basal cAMP level (± SD) of cultured medullary cells was 1.6 ± 0.72 pmol/μg DNA. AVP produced a significant increase (P < 0.01) in cAMP accumulation with a dose of $1 \times 10^{-11}$ M (2.5 ± 0.51 pmol/μg DNA) and the rate of increase was dose-dependent up to $1 \times 10^{-7}$ M (12.9 ± 0.78 pmol/μg DNA). In contrast, human PTH produced a significant increase (P < 0.05) in cAMP accumulation at a dose of 93.3 μg/ml (2.3 ± 0.43 pmol/μg DNA) without further increase at higher concentrations.

![Fig. 4.](image)

Response of human kidney cortical cultured cell cAMP levels to graded doses of salmon calcitonin (sCT) (○) and arginine-vasopressin (AVP) (●). Results are the mean ± SD of the cAMP response at each hormone dose. The shaded area represents two standard deviations of mean baseline cAMP levels.

Table 4.

<table>
<thead>
<tr>
<th>Bovine PTH (μg/ml)</th>
<th>cAMP* (pmol/10 min/mg protein)</th>
<th>Per cent increase above basal levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.1 ± 0.4</td>
<td>–</td>
</tr>
<tr>
<td>0.21</td>
<td>14.3 ± 0.3</td>
<td>42</td>
</tr>
<tr>
<td>0.43</td>
<td>19.6 ± 1.0</td>
<td>94</td>
</tr>
<tr>
<td>0.86</td>
<td>24.9 ± 1.8</td>
<td>146</td>
</tr>
<tr>
<td>1.72</td>
<td>30.7 ± 3.0</td>
<td>204</td>
</tr>
<tr>
<td>3.44</td>
<td>39.9 ± 1.9</td>
<td>295</td>
</tr>
<tr>
<td>6.88</td>
<td>38.6 ± 2.1</td>
<td>282</td>
</tr>
</tbody>
</table>

* Experiments in triplicate (mean ± SD).

Discussion

This paper addresses question of whether human kidney cortical cells in primary culture have certain advantages as an experimental model to test the biological activity of human PTH. The apparent uniformity of cell morphology observed by phase microscopy and the presence of cell growth in monolayer culture, at least for the first 10 days, suggest that this cell population could be homogeneous enough to be used for testing biological activity of PTH. In parallel with the increase in DNA content in dishes during the first period of culture, the baseline cAMP content of kidney cells doubled after 10 days. In contrast, the per cent PTH-stimulated cAMP accumulation increased progressively up to the fifth day of culture and declined slowly thereafter. This is either due to a partial de-differentiation of epithelial cells or to an increase of contaminating cells, such as fibroblasts. In view of this monolayer cells were used after 5 days of primary culture.

The response of human kidney cortical cells to bovine PTH (1–84) was compared to that of human kidney cortical purified plasma membranes. Bovine PTH (1–84) stimulated the adenylate cyclase activity of plasma membranes starting from a concentration of 0.4 μg/ml, in a way similar to that previously described by Di Bella et al. (1976). With kidney cortical cultured cells the minimal effective dose of bovine PTH (1–84) was 10-fold lower (i.e. 53.7 ng/ml), showing that the
sensitivity of intact isolated cells is greater than that of models such as acellular preparations or organ fragments. The cultured cell cAMP accumulation was rapidly stimulated by bovine PTH (1–84), with a significant increase above basal values after 5 min of incubation, and a plateau was reached after 30 min incubation. A similar quick response was observed when monolayer cells were stimulated with human PTH, purified by the medium of primary parathyroid cell cultures, and with bovine synthetic PTH (1–34).

Comparing the dose-response curves of the types of PTH used, a lower sensitivity to synthetic bovine PTH (1–34) was observed than with bovine PTH (1–34). The present findings agree with those described by Parsons et al. (1975) who evaluated the PTH effect on the adenylate cyclase activity of rat kidney cortical membrane; in contrast Arnaud et al. (1975) found that the adenylate cyclase of rat renal cortical membranes was more sensitive to bovine synthetic PTH (1–34) than to bovine PTH (1–34); recently the same group (Nissenson et al. 1981) found no difference between bovine PTH (1–84) and bovine synthetic PTH (1–34) using a guanyl nucleotide-amplified canine renal cortical adenylate cyclase. Nevertheless human kidney cortical cultured cells revealed a greater sensitivity to human 'culture' PTH than that to bovine PTH (1–84), showing that such an experimental model may be suitable for the study of hormone-cell interaction with respect to species specificity. On the other hand, when the minimal effective dose of human 'culture' PTH was used, it appeared to be of the same magnitude of other sensitive PTH bioassays, based on the adenylate cyclase-cAMP system stimulation, where an homologous substrate was also used (Heath & Zull 1980). However, the cytotoxic assay, based on the stimulation of glucose-6-phosphate-dehydrogenase activity of the distal convoluted tubule of the guinea pig, still appears to be the most sensitive method for detecting the biological activity of PTH (Chambers et al. 1978; Fenton et al. 1978). In addition Nissenson et al. (1981) found that canine renal membranes were a sensitive tool for evaluating biologically active human PTH when using, as an amplifier, guanylnucleotide in an adenylate cyclase assay.

When the effect of other peptide hormones on cAMP accumulation was studied in human kidney cortical cultured cells, AVP appeared to be a good stimulator of human kidney cortical cells, but with a potency and efficacy inferior to that of PTH. This can be explained by the presence of an AVP-sensitive adenylate cyclase in the thick ascending limb of the loop of Henle and in the cortical portion of the collecting tubule of the rat (Imbert-Teboul et al. 1978) and human (Chabardes et al. 1980) kidney, in partial disagreement with the observation of Chase & Aurbach (1968) who demonstrated that PTH and AVP stimulate adenyl cyclase at an anatomically separable area within the rat kidney. Human CT produced a poor effect on kidney cortical cell cAMP accumulation, while salmon CT was a slightly more potent stimulator. This observation is in agreement with the in vivo research of Caniggia et al. (1980), who observed minor changes in the renal production of cAMP after CT infusion. Finally, glucagon produced little significant increase of cAMP accumulation thereby confirming, in humans, the results of Marcus & Aurbach (1969) in rats.

Moreover, the specificity of PTH action for kidney cortical cells was confirmed by results obtained by us in kidney medullary cell cultures, where AVP was a more potent and efficacious stimulator of cAMP accumulation than in kidney cortical cells, and PTH exerted a weak or absent effect even at high concentrations.

In conclusion, even if human kidney cortical primary cultures are responsive to PTH at doses higher than circulating ones in normal subjects, they do show a greater biological species specificity for human PTH. For this reason and considering the lack of interference of serum protein in the assay, different from that found for other polypeptide hormones (Rapoport & Adams 1978), this model could be used to evaluate the biological activity of endogenous PTH in all situations where immunoreactive hormone levels are increased.

Acknowledgments

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References


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