Activation of the Parathyroid Hormone Receptor-Adenylate Cyclase System in Osteosarcoma Cells by a Human Renal Carcinoma Factor¹

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ABSTRACT

Human renal carcinoma cell line 786-0 elaborates a protein that is structurally and immunochemically distinct from parathyroid hormone (PTH) and that activates renal cortical adenylate cyclase via an interaction with the PTH receptor. Because of the high frequency of excessive bone resorption and resultant hypercalcemia in patients with malignant disease we evaluated the ability of this 786-0 cell factor to reproduce PTH action in bone-derived cells. The 786-0 factor as well as bovine PTH (BPTH) (1-34) and prostaglandin E₁ produced marked increases in cyclic adenosine 3':5'-monophosphate (cAMP) accumulation in the clonal rat osteosarcoma cell line UMR-106. A competitive antagonist of PTH action, [norleucine⁸, norleucine¹⁸, tyrosine³⁴]BPTH(1-34) and prostaglandin E₃ produced marked increases in cyclic derived cells. The 786-0 factor as well as bovine PTH (BPTH) (1-34) and prostaglandin E₁ produced marked increases in cyclic adenosine 3':5'-monophosphate (cAMP) accumulation in the clonal rat osteosarcoma cell line UMR-106. A competitive antagonist of PTH action, [norleucine⁸, norleucine¹⁸, tyrosine³⁴]BPTH(1-34)amide, blocked the cAMP stimulation produced by 786-0 factor and BPTH(1-34) but not that produced by prostaglandin E₁. In the presence of forskolin (0.1 μM) UMR-106 cells were extremely sensitive to 786-0 factor, showing significant increases in cAMP production at a concentration 10-fold less than that required to activate adenylate cyclase in renal membranes. In contrast UMR-106 cells were less sensitive to BPTH(1-34) than were renal membranes. This preferential increase in sensitivity to 786-0 factor was not seen in membranes prepared from UMR-106 cells suggesting the importance of cytosolic components. Six additional human genitourinary carcinoma cell lines were found to produce factors that increased cAMP levels in UMR-106 cells.

We conclude that 786-0 factor is a potent activator of the PTH receptor-adenylate cyclase system in these bone-derived cells. These findings are consistent with the view that cancer-associated hypercalcemia may frequently be attributable to tumor secretion of proteins (such as 786-0 factor) that are distinct from PTH but are capable of activating skeletal PTH receptors.

INTRODUCTION

A syndrome with features similar to those of primary hyperparathyroidism frequently occurs in patients with nonparathyroid cancers (1, 2). These features include elevated serum calcium caused by excessive bone resorption (3–5), decreased serum phosphate due to renal phosphate wasting (6), and increased urinary excretion of nephrogenous cAMP⁶ (6–8). Although it has been proposed that such tumors may secrete ectopic PTH or a closely related protein, serum levels of PTH measured by radioimmunoassay are generally low or normal in these individuals (6, 9, 10). Furthermore in a recent study Simpson et al. (11) failed to detect PTH mRNA in a variety of tumors associated with hypercalcemia. Conceivably, malignant cells may commonly secrete a distinct gene product that has a spectrum of biological effects overlapping that of PTH.

In an effort to elucidate the nature of this tumor-associated, PTH-like activity we have identified an established human renal carcinoma cell line, 786-0, that when grown as tumors in nude mice reproduces the syndrome of hypercalcemia, hypophosphatemia, and excessive bone resorption (12). The 786-0 cells originally isolated from a hypercalcemic patient produce a heat-stable, trypsin-sensitive factor that activates adenylate cyclase in canine renal cortical membranes. This presumably occurs via the PTH receptor since the competitive PTH antagonist [Nle⁸,Nle¹⁸,Tyr³⁴]BPTH(3-34)amide (13) blocks activation. Yet the 786-0 factor is distinguishable from PTH both by its larger molecular size and by its failure to react in four different radioimmunoassays, each of which is specific for a different region of the human PTH molecule. Stewart et al. (14) have described a similar factor in extracts of tumors from hypercalcemic patients.

In view of evidence that increased bone resorption is the cause of hypercalcemia in patients with cancer we evaluated the capacity of the PTH-like 786-0 cell factor to reproduce the action of PTH on bone-derived cells. We report that this factor is a potent activator of the PTH receptor-adenylate cyclase system in the clonal rat osteosarcoma cell line UMR-106 and that factors with similar activity are produced by a variety of human genitourinary carcinoma cell lines.

MATERIALS AND METHODS

Materials. We obtained BPTH(1-34) and [Nle⁸,Nle¹⁸,Tyr³⁴]BPTH(3-34)amide from Peninsula Laboratories (Belmont, CA), forskolin from Calbiochem (La Jolla, CA), [2,8-³H]cAMP from New England Nuclear (Boston, MA), and L-[⁶⁵S]methionine (1425 Ci/mmol) from Amersham (Arlington Heights, IL). All tissue culture media and additives were obtained from the Cell Culture Facility, University of California, San Francisco, CA. Other reagents were obtained from sources listed previously (12).

Cell Culture. Genitourinary carcinoma cell lines were established in culture and maintained according to published methods (15). The origin of these cell lines has been described previously (16). Line SK-LC-6 (formerly designated SK-luc-6) is an established anaplastic lung carcinoma cell line (17) kindly provided by Dr. J. Fugh (Sloan-Kettering Institute, Rye, NY). Renal carcinoma cell line 786-0 and other cell lines were maintained in a humidified atmosphere (5% CO₂ in air) in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% refiltered fetal bovine serum, 2 mm glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) and subcultured weekly by trypsinization. The clonal rat osteosarcoma cell line UMR-106 (18), kindly provided by Dr. T. J. Martin (Repatriation General Hospital, West Heidelberg, Australia), was maintained in minimal essential medium contain-
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ing 10% fetal bovine serum, 20 mM Hepes, pH 7.4, 2 mM glutamine, 2% nonessential amino acids, streptomycin (100 μg/ml), gentamicin (50 μg/ml), and fungizone (2.5 μg/ml). UMR-106 cells are known to have phenotypic characteristics of osteoblasts including high alkaline phosphatase activity and adenylyl cyclase responsiveness to PTH and prostaglandins (18).

Preparation of UMR Membranes. Confluent UMR cells (36 75-cm² flasks) were scraped into ice-cold buffer (0.25 M sucrose/liter mM EDTA/5 mM Tris-HCl, pH 7.5) and homogenized with 30 strokes of a tight glass Dounce homogenizer. Membranes were sedimented at 30,000 × g for 15 min and resuspended in fresh buffer (0.25 M sucrose/liter mM EDTA/5 mM Tris-HCl, pH 7.5) by rehomogenization (5 strokes). Membranes were stored at a protein concentration of 2.8 mg/ml at -80°C prior to assay.

PTH Bioassays. A protein fraction for bioassay was isolated from conditioned media essentially as described (12). Briefly, confluent cells were transferred to serum-free RPMI 1640 medium for 2 days. Conditioned media were desalted at 4°C on a 1.5- x 60-cm column of Bio-Gel P-4 (Bio-Rad Laboratories) from Bio-Gel P-4 (Bio-Rad Laboratories), formate form. PTH standards and unknown samples were assayed in triplicate wells; results are shown as mean ± SE. For determination of PTH-like biological activity of a sample in either bioassay, results at serial dilutions were averaged and the mean biological activity was reported as the concentration of BPTH(1-34) required to produce an equivalent response, e.g., as ng equivalent/ml.

Bio-synthetic Labeling of 786-0 Cells. Confluent 786-0 cells were incubated with [35S]methionine (0.2 mCi/ml) added. After 2 days conditioned medium containing 35S-labeled proteins was collected and desalted by chromatography on a column of Bio-Gel P-4, as described above. The protein fraction obtained in this way did not contain any detectable free, unincorporated [35S]methionine.

HPLC. The protein fraction of medium from biosynthetically labeled 786-0 cells was lyophilized to dryness and resuspended in 0.5 ml 0.5 M formic acid-0.14 M pyridine, pH 3.15 (Buffer A). An aliquot (0.25 ml) containing 45,000 cpm [35S]methionine-labeled protein was injected onto a 25-cm × 4.6-mm silica C-18 HPLC column (Vydac 218 TP5). Unadsorbed proteins were collected during a 10-min elution with Buffer A (flow rate, 1 ml/min; each fraction, 2 ml) after which a 60-min, linear 0-40% n-propyl alcohol gradient in Buffer A was initiated. Fractions were collected on ice, lyophilized overnight, and resuspended in 0.2 ml 10 mM acetic acid for bioassay of PTH-like activity.

RESULTS

Initial experiments demonstrated that a protein fraction from conditioned culture medium of 786-0 human renal carcinoma cells (hereafter designated 786-0 factor) increased cAMP levels in UMR-106 clonal rat osteosarcoma cells. Chart 1 shows the time course of cAMP accumulation in the presence of maximally effective concentrations of 786-0 factor or BPTH(1-34). The maximal cAMP response to each stimulus was obtained within 10-15 min and maintained for at least 30 min. Subsequent assays used 10-min incubations.

To evaluate the possibility that PTH and 786-0 factor might act on UMR-106 cells via a common receptor, we tested the effect of a competitive PTH antagonist analogue, [Nle⁶,Nle¹⁸,Tyr³⁴]BPTH(3-34)amide, on the cAMP response to BPTH(1-34) and 786-0 factor (Table 1). The analogue itself was found to be abortive agonist and it virtually abolished the stimulation of cAMP produced by just-maximally effective concentrations of 786-0 factor or BPTH(1-34). In contrast the analogue did not influence cAMP levels in the presence of 786-0 factor or BPTH(1-34) (data not shown). The data establish that 786-0 factor and BPTH(1-34) produced by just-maximally effective concentrations of 786-0 factor or BPTH(1-34). In contrast the analogue did not influence.

<table>
<thead>
<tr>
<th>UMR-106 cell cAMP (pmol/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>BPTH(1-34)</td>
</tr>
<tr>
<td>786-0 factor</td>
</tr>
<tr>
<td>Prostaglandin E₁</td>
</tr>
</tbody>
</table>

Table 1 Specific inhibition of 786-0 factor-stimulated UMR-106 cAMP levels by [Nle⁶,Nle¹⁸,Tyr³⁴]BPTH(3-34)amide

Concentration of Medium from 786-0 cells was measured following incubation with just-maximally effective concentrations of BPTH(1-34) (67 ng/ml), 786-0 factor, or prostaglandin E₁ (10 μg/ml). The competitive PTH antagonist [Nle⁶,Nle¹⁸,Tyr³⁴]BPTH(3-34)amide (67 ng/ml) was also added in parallel incubations. Each value is the mean ± SE of five replicate dishes.
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Chart 2. Reverse-phase HPLC of biosynthetically labeled, desalted conditioned medium from 786-0 cells. A, stimulation of cAMP production in osteosarcoma cells (C) and stimulation of adenylate cyclase in canine renal cortical plasma membranes (B). Results are expressed as the quantity of BPTH(1-34) required to produce an equivalent response. B, profiles of total protein (---) and of biosynthetically labeled protein (C) in the column fractions from A. eq., equivalent; n-propanol, n-propyl alcohol.

the cAMP response to a just-maximally effective concentration of prostaglandin E1.

Fractionation experiments were performed to determine whether the cAMP-stimulating activity in osteosarcoma cells and the previously demonstrated adenylate cyclase-stimulating activity in renal membranes might be intrinsic to the same protein in 786-0 factor preparations. These activities were found to copurify on reverse-phase HPLC (Chart 2A). Fractions with biological activity contained only a minute proportion (<5%) of the total medium protein as well as the biosynthetically labeled medium protein, demonstrating the efficacy of this HPLC system in the partial purification of 786-0 factor. Copurification of cAMP-stimulating activity and renal adenylate cyclase-stimulating activity was also observed on high resolution gel filtration chromatography on a column of Bio-Gel P-150 (not shown).

It should be noted that 786-0 factor was about 30-fold more potent in UMR-106 cells than in renal membranes when values are expressed in equivalents of BPTH(1-34). Parallel experiments in the two assay systems using the same dilutions of BPTH(1-34) and 786-0 factor demonstrate that the UMR-106 cell cAMP assay was about 10-fold more sensitive to 786-0 factor than was the renal adenylate cyclase assay (Chart 3). In contrast, UMR-106 cells were about threefold less sensitive than renal membranes to stimulation with BPTH(1-34). We consistently observed this difference in 20 experiments using 15 different preparations of 786-0 factor. One explanation for these findings is that 786-0 factor may be more stable than BPTH(1-34) in the UMR-106 cell cAMP assay. To examine this possibility the renal adenylate cyclase assay was used to determine residual biological activity in preparations of 786-0 factor and BPTH(1-34) that had been exposed to either assay condition (Table 2). About one-half the initial biological activity of both 786-0 factor and BPTH(1-34) remained after exposure to the renal adenylate cyclase assay conditions. Similar results were obtained after exposure of 786-0 factor to UMR-106 cAMP assay conditions, whereas somewhat less activity (23%) due to BPTH(1-34) was recovered.

To examine more directly the interaction between 786-0 factor and UMR-106 adenylate cyclase we compared the ability of 786-0 factor and UMR-106 adenylate cyclase to raise cAMP levels in the intact cell with its ability to stimulate adenylate cyclase in UMR-106 membranes. To facilitate this comparison adenylate cyclase activity in UMR-106 mem-

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Table 2
Effect of different assay conditions on biological activity of BPTH(1-34) and 786-0 factor

<table>
<thead>
<tr>
<th>Condition</th>
<th>Test substance</th>
<th>% bioactivity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal membranes</td>
<td>BPTH(1-34)</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>786-0 factor</td>
<td>47</td>
</tr>
<tr>
<td>UMR-106 cells</td>
<td>BPTH(1-34)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>786-0 factor</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 3
PTH-like bioactivity in conditioned media from human carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origin (carcinoma)</th>
<th>Renal adenylate cyclase assay</th>
<th>UMR-106 cell cAMP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>786-0</td>
<td>Renal cell</td>
<td>0.47</td>
<td>9.6</td>
</tr>
<tr>
<td>1072-F</td>
<td>Renal cell</td>
<td>0.33</td>
<td>5.6</td>
</tr>
<tr>
<td>639-V</td>
<td>Transitional cell</td>
<td>UND*</td>
<td>2.6</td>
</tr>
<tr>
<td>CAF-1</td>
<td>Renal cell</td>
<td>UND</td>
<td>1.1</td>
</tr>
<tr>
<td>647-V</td>
<td>Transitional cell</td>
<td>UND</td>
<td>0.8</td>
</tr>
<tr>
<td>769-P</td>
<td>Renal cell</td>
<td>UND</td>
<td>0.7</td>
</tr>
<tr>
<td>253-J</td>
<td>Transitional cell</td>
<td>UND</td>
<td>0.2</td>
</tr>
<tr>
<td>SK-LC-6</td>
<td>Anaplastic lung</td>
<td>UND</td>
<td>UND</td>
</tr>
<tr>
<td>V 382-D</td>
<td>Renal cell</td>
<td>UND</td>
<td>UND</td>
</tr>
</tbody>
</table>

* UND, undetectable, <0.05 and <0.15 in renal adenylate cyclase and UMR-106 cAMP assays, respectively.

DISCUSSION

The present studies demonstrate that 786-0 human renal carcinoma cells as well as a variety of other human genitourinary carcinoma cell lines produce a factor(s) that activates adenylate cyclase in the clonal rat osteosarcoma cell line UMR-106. Occupation of PTH receptors on these cells with high concentrations of [Nle8,Nle18,Tyr34]BPTH(3-34)amide, a competitive antagonist of PTH in renal and skeletal adenylate cyclase systems (13, 21, 22), blocked the increase in osteosarcoma cell cAMP produced by 786-0 factor or BPTH(1-34). In contrast stimulation of 4When forskolin was exduded, as in the experiment shown in Chart 4, UMR-106 cells were consistently found to be 2- to 3-fold less sensitive to both BPTH(1-34) and 786-0 factor than under standard assay conditions (0.1 μM forskolin), as shown in Chart 3.

branes was assayed without the addition of guanyl nucleotides and forskolin was omitted from the UMR-106 cell experiments. Representative experiment (Chart 4) the sensitivity to BPTH(1-34) was similar in UMR-106 cells and membranes, with half-maximal responses to BPTH(1-34) to 5.0 and 8.0 ng/ml, respectively. In contrast UMR-106 membranes were approximately 10-fold less sensitive to 786-0 factor than were UMR-106 cells.

Table 3 displays the results of UMR-106 cell cAMP assays of protein fractions from conditioned media from several different carcinoma cell lines. The only cell lines from patients known to have been hypercalcemic were 786-0 (15) and SK-LC-6 (17). However, unlike 786-0 cells (12) SK-LC-6 cells do not induce hypercalcemia when grown as large tumors in the nude mouse (data not shown) suggesting that they may have ceased to secrete a mediator of hypercalcemia. It is of interest that media from several tumors unassociated with hypercalcemia appear to contain small amounts of PTH-like biological activity. Only the two media samples with the greatest bioactivity (786-0 and 1072-F) were detected in the renal adenylate cyclase assay, as would be predicted if that assay is generally 10-fold less sensitive to tumor peptides than is the UMR-106 cell cAMP assay. Stimulation of renal adenylate cyclase by the protein fraction of 1072-F medium was eliminated by prior exposure to trypsin and was blocked by the PTH antagonist [Nle8,Nle18,Tyr34]BPTH(3-34)amide (data not shown). These results are consistent with the interpretation that the bioactive principle in 1072-F medium as in 786-0 medium is a peptide that activates adenylate cyclase as the result of an interaction with the PTH receptor. The sample from 1072-F medium like that from 786-0 medium was 20- to 30-fold more potent in the osteosarcoma cells than in renal membranes (when values are expressed in PTH equivalents).

The present studies demonstrate that 786-0 human renal carcinoma cells as well as a variety of other human genitourinary carcinoma cell lines produce a factor(s) that activates adenylate cyclase in the clonal rat osteosarcoma cell line UMR-106. Occupation of PTH receptors on these cells with high concentrations of [Nle8,Nle18,Tyr34]BPTH(3-34)amide, a competitive antagonist of PTH in renal and skeletal adenylate cyclase systems (13, 21, 22), blocked the increase in osteosarcoma cell cAMP produced by 786-0 factor or BPTH(1-34). In contrast stimulation of

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cAMP levels by prostaglandin E\(_1\) was not inhibited by [Nle\(^8\),Nle\(^{18}\),Tyr\(^34\)]BPTH(3-34)amide. These results provide strong, if indirect, evidence that 786-0 factor must occupy the PTH receptor in order to activate adenylate cyclase in osteosarcoma cells. The 786-0 factor appears to be identical to the previously reported protein that stimulates PTH-responsive adenylate cyclase in renal membranes (12) since (a) the two activities are copurified by HPLC and gel filtration chromatography and (b) a relatively constant relationship between biological activities in the two systems was seen in 15 lots of spent culture medium. However, it is formally possible that the two activities are intrinsic to separate proteins, since HPLC and gel filtration fractions with peak PTH-like bioactivity displayed multiple protein components upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (not shown). Despite its PTH-like bioactivity 786-0 factor is a larger molecule (by gel filtration chromatography) than PTH and fails to cross-react in multiple sensitive PTH radiimmunoassays (12).

Our results indicate that PTH and 786-0 factor also differ in their biological activities. Thus BPTH(1-34) is about 3-fold less potent in the UMR-106 cell CAMP assay than in the 5'-guanylimidodiphosphate-amplified canine renal adenylate cyclase assay. Conversely the tumor peptides are 10-fold more potent in the UMR-106 assay. This enhanced sensitivity permitted us to detect "PTH-like" bioactivity in conditioned media of seven human genitourinary carcinoma cell lines; bioactivity was detectable in only two using the renal adenylate cyclase assay. The basis for these differences in relative potency between 786-0 factor and PTH remains uncertain. Our results indicate that differences in the stability of PTH and 786-0 factor under the assay conditions described cannot fully account for observed potency differences. Perhaps PTH receptors in UMR-106 cells have a greater relative affinity for 786-0 factor and related tumor peptides than do PTH receptors in canine renal membranes. If so the selective decrease in sensitivity to 786-0 factor observed in membranes from UMR-106 cells (Chart 4) could be due to subtle alterations in the PTH receptor during membrane preparation resulting in diminished affinity for 786-0 factor but not for PTH. Competitive binding studies in these systems using \(^{125}\)IJBPTH(1-34) will be needed to substantiate this idea. Alternatively in some systems cytosolic factors are known to alter receptor-adenylate cyclase coupling (23) and these might preferentially enhance the coupling to adenylate cyclase of the PTH receptor occupied by 786-0 factor.

Excessive osteoclastic bone resorption appears to account for the hypercalcemia associated with a variety of cancers in humans (3–5). Thus nude mice bearing 786-0 tumors develop marked hypercalcemia (12) which persists despite severe restriction of dietary calcium. The bones of these animals display histomorphometric evidence of increased osteoclastic bone resorption (24). Thus the finding that 786-0 factor activates the receptor for PTH, a potent bone-resorbing hormone, in bone-derived cells and in renal membranes supports the concept that 786-0 factor may be representative of agents that are etiologically related to the hypercalcemia of cancer. The UMR-106 rat osteosarcoma cells used in the present studies have phenotypic properties of osteoblasts including responsiveness to PTH (18).

Although the precise mechanisms whereby PTH increases bone resorption are poorly understood, there is little evidence that PTH directly stimulates osteoclasts. Hence it has been proposed that PTH via its adenylate cyclase-coupled receptor on osteoblasts may produce alterations in osteoblast morphology (25) or metabolism that indirectly augment osteoclastic bone resorption (26). Activation of the osteoblastic PTH receptor by tumor-derived proteins such as 786-0 factor would be expected to initiate the same cascade of events leading to bone resorption.

It is conceivable that tumor secretion in vitro of PTH-like proteins such as 786-0 factor represents an epiphenomenon unrelated to the development of hypercalcemia in vivo. However, it is of interest that of the cell lines we examined 786-0 produces the largest amount of PTH-like bioactivity in vitro and causes severe hypercalcemia in vivo (12) whereas 253-J (transitional cell carcinoma) and SK-LC-6 (anaplastic lung carcinoma) produce little or no PTH-like bioactivity in vitro and fail to induce hypercalcemia when grown as large tumors in nude mice. Because of the genotypic and phenotypic changes during cell culture it is problematic to correlate properties of the individual cell lines with those of the original tumor cells. Thus SK-LC-6, although derived from a hypercalcemia patient, failed to produce hypercalcemia in vivo. Perhaps such changes during continuous culture also account for the large amount of PTH-like bioactivity produced by a renal carcinoma cell line (1072-F) originating from a normal carcinoma patient. We are currently attempting to grow these cells as tumors in nude animals to determine whether they have acquired the ability to produce hypercalcemia in vivo.

While the work described here was in progress Rodan et al. (27) reported that conditioned media from cell cultures of two animal tumors and one human tumor that produce hypercalcemia as well as an extract of a lung tumor from a hypercalcemia patient stimulated cAMP accumulation in ROS 17/2.8 rat osteosarcoma cells. These results together with our own support the concept that tumor production of factors with PTH-like bioactivity is a common occurrence. Such factors are distinct from PTH both immunochemically and by their larger molecular size, yet they appear to exert their PTH-like actions by binding to renal and skeletal PTH receptors. Whether these tumor proteins in purified form will reproduce the hypercalcemia and bone-resorbing activities of PTH in vivo remains to be established.

ACKNOWLEDGMENTS

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REFERENCES


* From the present data it cannot be determined whether the small amounts of cAMP-stimulating activity in 253-J and other media reflect binding of a medium factor to the PTH receptor. When stimulation of cAMP is not marked, the PTH antagonist [Nle\(^8\),Nle\(^{18}\),Tyr\(^34\)]BPTH(3-34)amide cannot readily be used to test specificity for the PTH receptor because of its distinct, albeit weak, agonist activity.

G. J. Strewler, R. D. Williams, and R. A. Nissenson, unpublished data.


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