Identification and characterization of parathyroid hormone receptors on dog kidney, human kidney, chick bone and human dermal fibroblast

A comparative study of functional and structural properties

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To evaluate the functional and structural characteristics of the parathyroid hormone (PTH) receptors on different tissues and the possible heterogeneity in structure and function, PTH receptors on dog kidney membrane, human kidney membrane, chick bone cell membrane and human dermal fibroblast membrane were evaluated. The results showed that human kidney plasma membrane, canine kidney plasma membrane and chick bone cell membrane possess one single class of PTH receptor with a K_d (dissociation constant) of 1–5 nM and an IC_{50} also of 1–5 nM. The number of binding sites was 800 fmol per mg of protein for chick bone cell particulate membrane, 1–5 pmol per mg of protein for human kidney plasma membrane and 2.2 pmol per mg of protein for dog kidney plasma membrane. Photoaffinity labelling identified a major binding component with a molecular mass of 70 kDa in all three types of membrane. The plasma membrane fraction from human dermal fibroblast contained two different binding sites for PTH with high ($K_d = 2$ nM) and low ($K_d = 580$ nM) affinities respectively. The IC_{50} for the adenylate cyclase is about 2 nM, which is similar to the K_d of the high-affinity site. Photoaffinity labelling also demonstrated a major binding component with a molecular weight of 70 kDa. We conclude that structural and functional similarity exists among the PTH receptors present on chick bone cell membrane, dog kidney membrane and human kidney membrane. The human dermal fibroblast possesses two different binding sites, one of which is coupled to adenylate cyclase.

INTRODUCTION

Using a classical radioligand binding assay, parathyroid hormone (PTH) receptors have been identified on chicken (Nissenson & Arnaud, 1979; McKee & Murray, 1985), rat (Chansel *et al.*, 1977), dog (Segre *et al.*, 1979; Teitelbaum *et al.*, 1982), bovine (Zull *et al.*, 1977) and rabbit (Kremer *et al.*, 1982) bone and kidney tissues. However, structural identification using photoaffinity labelling techniques has been performed mainly on dog kidney membrane (Coltrera *et al.*, 1981; Draper *et al.*, 1982; Goldring *et al.*, 1984; Nissenson *et al.*, 1987). Despite the above information on animal models, the functional and structural characteristics of human PTH receptors have not yet been defined. In this study, the PTH receptors in four different types of membrane preparations were evaluated in order to assess the similarity of functional and structural characteristics across different species and tissues.

MATERIALS AND METHODS

Materials

Synthetic human bPTH-(1–34) and bovine bPTH-(1–34) were prepared by Bachem Fine Chemicals (Torrance, CA, U.S.A.) by the solid-phase procedure as reported by Merrifield (1962) and had potencies exceeding 4000 U/mg. Cyclic AMP and 3-isobutylmethylxanthine were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and *N*-hydroxysuccinimidyl-

4-azidobenzoate (HSAb) was from the Pierce Chemical Corp. (Rockford, IL, U.S.A.). [α - 32 P]ATP was purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.); [3 H]cAMP from New England Nuclear Co. Ltd. (Boston, MA, U.S.A.); and GTP and Gpp(NH)p from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Bovine serum albumin fraction V was purchased from Miles Laboratories, Inc. (Elkhart, IN, U.S.A.). All other reagents were obtained from standard laboratory suppliers.

Isolation of plasma membrane fraction from human kidney

Fresh human kidney tissue was obtained and processed immediately after operation. The tissue was part of the total or partial nephrectomy specimen and surgical waste, and was obtained with consent from the patients. The kidney specimens were sliced longitudinally and the cortical tissues were dissected away from capsular tissue and fat. The pieces of cortical tissue were homogenized with a Polytron homogenizer and Teflon pestle. The homogenate was centrifuged at 2000 *g* for 20 min and, after resuspension, was recentrifuged at 14400 *g* for 20 min. Finally, the sedimented portion was recentrifuged at 22000 *g* for 15 min. This sedimented the plasma membranes as the top fluffy layer and the mitochondrial membranes as the bottom compact layer. The plasma membrane fraction was washed and resuspended three times before sorting at -80°C .

Abbreviations used: PTH, parathyroid hormone; HSAb, *N*-hydroxysuccinimidyl-4-azidobenzoate.

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Isolation of canine renal plasma membrane

Canine renal plasma membrane was prepared by the method of Fitzpatrick *et al.* (1969).

Isolation of human dermal fibroblast plasma membrane

Punch biopsy was performed on the extensor surface of the arm of a 29-year-old man. Human dermal fibroblasts were obtained from primary tissue explant culture. The fibroblasts were subsequently grown in Dulbecco's Modified Eagle's Medium H21, supplemented with 5% fetal calf serum, 2 mM-glutamine and 100 U of both penicillin and streptomycin/ml. Plasma membrane fractions were prepared from confluent cultures at passages 1–2 by the hypo-osmotic lysis method (Kartner *et al.*, 1977).

Isolation of chick bone cell membrane fractions

Chick bone cells were prepared from fertilized chicken eggs at the 10th day of embryo development as previously described (Silve *et al.*, 1982; Pliam *et al.*, 1982). Confluent cultures of chick bone cells in 75 ml flasks were washed three times with ice-cold phosphate-buffered saline (Dulbecco's) and then scraped out with a rubber policeman into 5 ml of 0.25 mM-sucrose/5 mM-Tris/HCl/1 mM-EDTA, pH 7.5 (SET buffer). The combined cell scrapings were homogenized on ice in a Dounce homogenizer using a tight pestle. This homogenate was centrifuged at 33000 *g* for 10 min and the pellet was resuspended in SET buffer. The final suspension was stored at -80°C . The yield was about 15–20% of the total cellular protein.

Preparation of electrolytically ^{125}I -labelled bPTH-(1–34)

bPTH-(1–34) was radiolabelled using an electrolytic iodination procedure as previously described by Nissenson *et al.* (1985).

Purification of ^{125}I -bPTH-(1–34)

After electrolytic labelling, the peptides were separated by h.p.l.c. The labelled bPTH-(1–34) was loaded onto a Waters C-18 Bondapak column equilibrated with 0.1% trifluoroacetic acid in water. The peptides were eluted with a 0–95% gradient of *N*-propanol of 0.1% trifluoroacetic acid.

Two major peaks of radioactivity were eluted from the reverse-phase C-18 Bondapak column at fractions 30–40. The second peak, which was eluted at 28% *N*-propanol, was used for subsequent experiments because it possessed more biological activity as assessed by binding to PTH receptors on dog renal plasma membrane. The cold hormone was eluted at about 37% *N*-propanol. Before storing at -20°C , the fractions in the second peak were pooled and chromatographed on Sephadex LH-20 columns with 10 mM-acetic acid/0.1% bovine serum albumin as eluant for solvent exchange. The total yield of ^{125}I -bPTH-(1–34) was approx. 0.1 mCi and the specific activity was about 50 $\mu\text{Ci}/\mu\text{g}$.

Measurement of ^{125}I -bPTH-(1–34) binding in membrane fractions

The binding assays for the four types of membrane were performed with the following method. The binding assays were done in a final volume of 100 μl containing 50 mM-Tris/HCl/50 mM-Hepes (pH 7.5)/2.0 mM-

MgCl_2 /0.1% bovine serum albumin, plus approx. 4 fmol of purified ^{125}I -bPTH-(1–34), and unlabelled bPTH-(1–34) or other peptides. The binding reaction was initiated by adding 25 μg of the plasma membrane. After 60 min incubation at 30°C , the sample was diluted with 1.0 ml of ice-cold washing buffer containing 0.1% bovine serum albumin/2.0 mM- MgCl_2 /25 mM-Tris/HCl (pH 7.5)/125 mM-NaCl. The samples were kept at 0°C afterwards and centrifuged for 10 min at 0°C to separate bound from free ^{125}I -bPTH-(1–34). After removing the supernatant, the microfuge tubes with pellets at the bottom were clipped and counted. Blank binding to microfuge tubes in the absence of plasma membranes was less than 0.5% of the added ^{125}I -bPTH-(1–34).

Adenylate cyclase assay of membrane fractions

Adenylate cyclase activities of membranes were studied by measuring the conversion of [α - ^{32}P]ATP into cyclic [^{32}P]AMP as described by Nissenson (1983).

Photolysis of plasma membrane fractions

Approx. 0.3 μCi of ^{125}I -bPTH-(1–34) was incubated for 90 min with plasma membrane fractions (125 μg) in 1.0 ml of a solution consisting of 50 mM-Tris/HCl/2 mM- MgCl_2 /0.1% bovine serum albumin/50 mM-Hepes (pH 7.5) in a microfuge tube. At the end of the incubation, incubates were microfuged at 4°C . The pellets were resuspended in 1.0 ml of ice-cold 50 mM-sodium phosphate with 0.1% bovine serum albumin (pH 7.6). After centrifugation, the pellets were washed four times with 50 mM-sodium phosphate without bovine serum albumin. The final pellet was resuspended in 350 μl of 50 mM-sodium phosphate. The heterofunctional cross-linking reagent, HSAb (45.5 μg), was added in the dark, and the incubation was carried out for 10 min at 4°C in the dark. Amino-group derivatization was terminated by the addition of 7 μl of 2 M-Tris/HCl, and the reaction mixture was transferred to 24-well cluster plates and photolysis was performed for 20 min at 4°C using a Blak-Ray lamp (emission maximum 365 nm) at a distance of 8 cm. After washing, the membranes were resuspended in SDS/polyacrylamide-gel electrophoresis sample buffer containing 10% β -mercaptoethanol for electrophoresis according to Laemmli (1970). SDS/polyacrylamide-gel electrophoresis was performed using 5–15% acrylamide gradient gels, under reducing conditions unless otherwise indicated.

Statistical analysis

Unless otherwise specified, the data were obtained from experiments performed in triplicate. The data points represent the means $\pm$ S.E.M. of triplicate assays for each condition in the cell population. The significance of difference between groups was determined by Student's *t* test as well as by the Analysis of Variance. A *P* value of less than 0.05 is taken as significant.

The K_d for PTH binding was determined by Scatchard analysis (Scatchard, 1949) of the data obtained from competitive binding experiments using ^{125}I -bPTH-(1–34) and various concentrations of unlabelled bPTH-(1–34).

RESULTS

Binding of ^{125}I -bPTH-(1–34) and activation of adenylate cyclase in the membrane preparation

Fig. 1 shows the results obtained with canine renal

Fig. 1. (a) Inhibition of ^{125}I -bPTH-(1-34) binding by unlabelled bPTH-(1-34), and (b) dose-response stimulation of cyclic AMP generation by bPTH-(1-34), in plasma membrane fractions obtained from dog renal cortex

(a) Plasma membrane fractions from dog renal cortex were incubated with ^{125}I -bPTH(1-34) and increasing concentrations of unlabelled bPTH-(1-34) under standard conditions, as specified in the Materials and methods section. Binding is expressed as the percentage of bound counts divided by total counts. Each point is the mean $\pm$ S.E.M. of three separate experiments. Inset: Scatchard analysis of the binding data. $K_d = 4.5 \pm 0.7$ nM; $B_{\max} = 54.4 \pm 8.2$ fmol/25 μg of protein. (b) Adenylate cyclase activity was measured as described in the Materials and methods section.

cortical membrane. The concentration of PTH required for half-maximal inhibition of ^{125}I -bPTH-(1-34) binding was about 4–5 nM (Fig. 1a). Scatchard analysis (inset) shows the presence of one single site with a dissociation constant (K_d) of 4.5 ± 0.7 nM and a maximal binding capacity $B_{\max}$ of 54.5 ± 8.2 fmol/25 μg of protein (about 2.18 pmol per mg of protein). The measurement of adenylate cyclase in canine renal cortical membrane demonstrated a 5-fold increase in activity after stimulation by PTH (Fig. 1b) and the concentration for half-maximal activation (IC_{50}) was about 1.1 nM.

Results obtained with human renal cortical membranes were similar to those of canine renal cortical membrane. The maximal binding of ^{125}I -bPTH-(1-34) to the plasma membrane preparation was $7.5 \pm 0.7\%$. The concentration required for half-maximal displacement of binding was 4.9 nM. Scatchard analysis shows the presence of one single site with a K_d of 4.9 ± 0.6 nM and a $B_{\max}$ of 37.8 ± 5.3 fmol/25 μg of protein (about 1.51 pmol per mg of protein). Activation of adenylate cyclase by PTH increased the activity from 149 ± 52 pmol of cyclic

Fig. 2. (a) Displacement experiments and (b) dose-response stimulation of cyclic AMP generation by bPTH-(1-34), in plasma membrane fractions obtained from human dermal fibroblasts

Inset: Scatchard analysis of the binding data; B/F, bound to free ratio. $K_{d1} = 2.07 \pm 1.42$ nM; $B_{\max,1} = 3.1 \pm 1.9$ fmol/25 μg of protein; $K_{d2} = 579 \pm 81$ nM; $B_{\max,2} = 694 \pm 92$ fmol/25 μg of protein.

AMP/30 min per mg of protein to 550 pmol. The IC_{50} was about 1.4 nM.

The results obtained in membrane fractions prepared from chick bone cells were also comparable to those of canine renal cortical membrane, except that the maximal binding of ^{125}I -bPTH-(1-34) to 25 μg of the membrane fractions was only $3.9 \pm 0.3\%$. The concentration required for half-maximal displacement was 43 nM. Scatchard analysis demonstrates one single site with a K_d of 4.3 ± 0.7 nM and a $B_{\max}$ of 19.5 ± 2.9 fmol/25 μg of protein. PTH increased the adenylate cyclase activity from the basal level of 96 ± 52 pmol of cyclic AMP/30 min per mg of protein to about 400 pmol/30 min per mg of protein. The IC_{50} , which was 1.5 nM, is also comparable to the K_d .

Fig. 2 shows the results obtained in plasma membrane fractions prepared from human dermal fibroblasts. The maximal binding of ^{125}I -bPTH-(1-34) to the membranes was $3.1 \pm 0.2\%$. There were two distinct phases of displacement, with a plateau between them. This shows that there are two receptors, with high and low affinities respectively (Fig. 2a). Scatchard analysis (inset) shows that the high affinity site has a K_{d1} of 2.07 ± 1.42 nM and a $B_{\max,1}$ of 3.1 ± 1.9 fmol/25 μg of protein (about 76 fmol per mg of protein). The low-affinity site has a K_{d2} of 579 ± 81 nM and a $B_{\max,2}$ of 694 ± 92 fmol/25 μg of protein (about 28 pmol per mg of protein). Measurement of adenylate cyclase demonstrated an increase in activity

Fig. 3. Photoaffinity labelling of PTH receptor on dog kidney plasma membrane

The major component labelled was a 70 kDa protein. Lane 1, 0 ng of bPTH-(1-34)/ml; lane 2, 5 ng/ml; lane 3, 20 ng/ml; lane 4, 100 ng/ml.

from 312 ± 104 pmol of cyclic AMP/30 min per 25 μ g of protein to about 750 pmol of cyclic AMP/30 min per 25 μ g of protein (Fig. 2b). The concentration of PTH required for half-maximal stimulation of adenylate cyclase was 2.2 nM, which is similar to the K_d of the high-affinity site.

Photoaffinity labelling of the PTH receptors on the membrane preparation

Figs. 3 and 4 show the results of photoaffinity labelling with electrolytically-labelled I^{125} -bPTH-(1-34).

In dog renal plasma membranes (Fig. 3), the major component labelled was a 70 kDa protein. In human renal cortical plasma membranes, the component labelled was similar to that in dog kidney membrane in molecular mass (70 kDa). As a control, the human renal medullary plasma membranes were not labelled at all. In chick bone cell membranes, the major component linked to I^{125} -bPTH-(1-34) after photoaffinity labelling was also a 70 kDa protein. The labelling of this component in the three different membranes can be inhibited with 200 ng of bPTH-(1-34)/ml.

In the plasma membrane fractions prepared from human dermal fibroblasts, the major component labelled is also a 70 kDa protein. In addition, a few different components with molecular masses between 30 and 70 kDa were labelled (Fig. 4). Displacement with 100 ng of PTH/ml was incomplete, while 5 μ g/ml completely displaced all visible binding.

Fig. 4. Photoaffinity labelling of PTH receptor on plasma membrane fractions prepared from human dermal fibroblasts

The major component labelled was also a 70 kDa protein. In addition, a few different components with molecular masses between 30 and 70 kDa were also labelled. Lane 1, 0 ng of bPTH-(1-34)/ml; lane 2, 100 ng/ml; lane 3, 5 μ g/ml.

DISCUSSION

The results show that human kidney plasma membrane, canine kidney plasma membrane and chick bone cell membrane possess a single class of PTH receptor with a dissociation constant of 1–5 nM. The K_d for I^{125} -bPTH-(1-34) binding is comparable to the K_m for PTH stimulation of adenylate cyclase in the membrane fractions. Scatchard analysis in these three systems shows that PTH binds to a single class of receptors which are present in different amounts. In membrane fractions from chick bone cells, there were 800 fmol/mg of membrane protein. In canine kidney and human kidney plasma membrane, there were about 2.18 pmol and 1.51 pmol of receptor/mg of membrane protein respectively. The results show that the PTH receptors on chick bone, human renal cortex and canine renal cortex are similar. On the other hand, binding experiments on human fibroblast plasma membrane demonstrated two different binding sites with high and low affinities. It has been shown that intact human dermal fibroblasts bound I^{125} -bPTH-(1-34) in a specific, saturable, time- and temperature-dependent manner (Pun *et al.*, 1986). The concentration of PTH required for half-maximal displacement of I^{125} -bPTH-(1-34) from the high-affinity site

is similar to the concentration of hormone which stimulates adenylate cyclase half-maximally. In addition, it is comparable to the concentration of PTH required for half-maximal displacement of PTH binding to human and dog kidney plasma membrane, chick bone cell membrane, bone cells (Demay *et al.*, 1985) and an established renal cell line (McKee & Murray, 1985).

While the concordance between the K_d for ^{125}I -bPTH-(1-34) binding to the high-affinity site and the IC_{50} for activation of adenylate cyclase indicates that the high-affinity site is coupled to adenylate cyclase, the physiological significance of the low-affinity site is unknown. The detection of two species of PTH receptor in this as well as other studies (Demay *et al.*, 1985; McKee & Murray, 1985) agrees with the physiological observation that there may be at least two different types of PTH receptors coupled to different second messengers. Photoaffinity labelling of PTH receptors on dog kidney membrane has been performed with several photoaffinity labels (Coltrera *et al.*, 1981; Draper *et al.*, 1982; Goldring *et al.*, 1984; Nissenson *et al.*, 1987). Our results obtained with the heterobifunctional, photoreactive reagent HSAb were in general agreement with previous reports. Using similar techniques on tissues with unknown properties, the PTH receptors on human kidney membrane, chick bone cell membrane and human fibroblast membrane were isolated and characterized. The structural similarity among the PTH receptors on dog kidney membrane, human kidney membrane, chick bone cell membrane and human fibroblast membrane is evidenced by the demonstration of major binding protein as the 70 kDa component in all membranes. The ready inhibition of labelling at a level of bPTH-(1-34) about 10-fold greater than the K_d of the receptors shows that the characteristics of binding to this 70 kDa protein are concordant with those observed in the binding experiments. In plasma membrane fractions of human dermal fibroblasts, the major component labelled is also a 70 kDa protein. In addition, a few minor components of smaller molecular mass are also labelled, especially in human dermal fibroblasts. These are also present to a lesser extent in other membranes. They may represent a second binding site for PTH which is present in various amounts in different tissues (Demay *et al.*, 1985; McKee & Murray, 1985).

To conclude, the results of this study show that there is structural and functional similarity among the PTH receptors present on chick bone membrane, dog kidney membrane and human kidney membrane. The human dermal fibroblast possesses two different binding sites, one of which is coupled to adenylate cyclase.

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