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Effects of Parathyroid Hormone on the Fluidity of the Plasma Membrane Vesicles of Cultured Osteoblasts

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Intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) were used to investigate the effects of parathyroid hormone (PTH) on the bulk bilayer fluidity of the plasma membrane vesicles isolated from cultured osteoblasts (OB-PMV). In a dose-dependent manner, rat PTH-(1-34) [rPTH-(1-34)] increased the excimer to monomer fluorescence intensity ratio (I'/I) of Py-3-Py and decreased the anisotropy (r) of DPH in OB-PMV. This indicates that PTH increased both the lateral and rotational diffusion of the probes in OB-PMV. Selective quenching of DPH fluorescence by trinitrophenyl groups was utilized to examine the transbilayer fluidity asymmetry of OB-PMV. The anisotropy, limiting anisotropy, and order parameter of DPH in the inner monolayer were 0.024, 0.032, and 0.062 greater than calculated for the outer monolayer of OB-PMV. Selective quenching of DPH fluorescence by trinitrophenyl groups was also utilized to examine the transbilayer effects of PTH on the fluidity of OB-PMV. rPTH-(1-34) had a greater fluidizing effect on the outer monolayer as compared to the inner monolayer of OB-PMV. Thus, it has been proven that PTH exhibits a selective rather than nonselective fluidizing effect within transbilayer domains of OB-PMV.

key words: parathyroid hormone, membrane fluidity, osteoblasts, fluorescent probe technique

INTRODUCTION

With an increase in people's life span, osteoporosis is progressively recognized as a major global health issue. The maintenance of normal, healthy bone requires the coupling of bone formation by osteoblasts to bone resorption by osteoclasts such that the two processes are balanced. All currently approved therapies for osteoporosis (e.g., estrogen, bisphosphonate, calcitonin and selective estrogen receptor modulators) are anti-resorptive agents that act on osteoclasts to prevent further bone loss [1-3]. Although parathyroid hormone (PTH) is best recognized as the key hormone which promotes bone resorption and elevates blood calcium, the bone-building action of PTH was noted in the 1930s [4]. Today, there is a wealth of preclinical data showing that daily intermittent administration of low dose PTH increases mechanical strength and mass in trabecular and cortical bone in animals and humans [5-8]. At present, PTH and its N-terminal peptide analogs are considered as the most promising bone anabolic agent capable of building mechanically strong new bone in patients with established osteoporosis [5-8].

Most of the biological activity of PTH is thought to be mediated by binding of its amino terminus to the PTH/PTH-related protein (PTHrP) receptor which is embedded in the plasma membranes of osteoblasts, resulting in downstream signaling pathways [9-11]. The PTH/PTHrP receptor is a class II G protein coupled receptor [12], which couples strongly to the adenylyl cyclase-protein kinase A signaling pathway, and generally less robustly to the phospholipase C-protein kinase C-intracellular Ca^{2+} signaling pathway [9]. However, most of cellular and molecular physiology underlying the anabolic effects of PTH is still unclear, including the molecular basis for the observation that continuous high-dose administration of PTH causes predominantly osteoclastic resorption, whereas lower intermittent doses elicit anabolic effects.

A large, diverse collection of physiological agonists including hormones produces the alterations in membrane fluidity as well as their specific ligand-receptor interaction [13-17]. The present study was undertaken to determine if PTH also exerts nonspecific actions on the lipids of plasma membrane vesicles isolated from cultured osteoblasts (OB-PMV). Using intramolecular excimerization of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), the effects of rat PTH-(1-34) [rPTH-(1-34)] on the bulk bilayer fluidity of the OB-PMV were examined. Also, selective quenching of DPH fluorescence by trinitrophenyl groups was utilized to examine the effects of rPTH-(1-34) on the individual monolayer structure of OB-PMV. The results indicate that alterations in membrane fluidity may also play a role in PTH action.

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MATERIALS AND METHODS

Materials rPTH-(1-34), bovine serum albumin (BSA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and buffers were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA (0.05% trypsin, 0.02% EDTA), collagenase, trypsin, and phosphate-buffered saline (PBS; 9 g/*l* NaCl, 0.144 g/*l* KH_2PO_4, 0.795 g/*l* $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) from Gibco BRL (Life Technologies, Grand Island, NY, USA); and DPH and Py-3-Py from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest quality available, and water was deionized with a Milli-Q system.

Cell culture Freshly isolated osteoblasts were obtained from calvariae of newborn Sprague-Dawley rats [18]. Fourteen calvariae were dissected out, and all adhering soft tissue was removed. The calvariae were cut into pieces and subjected to six sequential 20-min digestions in PBS containing 0.1% collagenase, 0.05% trypsin, and 4 mM EDTA. Cells from a pool of populations 4-6 were cultured in DMEM supplemented with 10% FBS, 50 U/m*l* penicillin, and 50 μg/m*l* streptomycin. After reaching confluence, cells were harvested by trypsin-EDTA treatment and passaged at a ratio of 1:4. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 with changing medium every 3-4 days.

TNBS labelling reactions TNBS labeling reactions were performed by the procedure of Yun and Kang [19]. The cultured cells were gently suspended in 2 mM TNBS+buffer A or buffer A alone. Buffer A was composed of 30 mM NaCl, 120 mM $NaHCO_3$, 11 mM glucose, 2% BSA, pH 8.5. To assure complete exposure of all outer monolayers to TNBS, the cells were passed slowly through a glass Dounce homogenizer (3 up and down strokes). Unless otherwise specified, the treatment was carried out at 4°C for 40 min. The TNBS labeling reaction was terminated by adding 1% BSA in PBS. The entire suspension was then sedimented at 1,100× g for 5 min, and preparation of plasma membrane vesicles was performed as follows.

Isolation of the plasma membrane vesicles OB-PMV were isolated by a modification of the methods of Bütler *et al.* [20] and Mitchell *et al.* [21]. All steps were carried out at 0-4°C. Briefly, the cultured cells were washed twice in ice-cold PBS, followed by hypotonic lysis in 5 mM Tris·HCl –1 mM EDTA, pH 7.4. Lysed cells and buffer were removed from the flasks and homogenized with 30 strokes in a glass Dounce homogenizer, and nuclei and cell debris were sedimented for 20 min at 2,000× g. The supernatant was subsequently centrifuged for 60 min at 100,000× g using a SW40 rotor. The pellet was resuspended in PBS and layered over a 40% (w/v) sucrose in PBS. After centrifugation for 45 min at 40,000× g in a SW40 rotor, the material at the buffer/sucrose interface was collected, diluted with PBS and recentrifuged at 100,000× g for 45 min. The final pellet was resuspended in PBS, divided into small aliquots, rapidly frozen in liquid nitrogen, and stored at −70°C. The purity was evaluated by morphological and enzymatic standards. Electron microscopic examination showed that the membranes were in vesicular form. The purity of OB-PMV was also confirmed by monitoring the specific activities of marker enzymes. The specific activities of Na,K-ATPase and 5′-nucleotidase were about 5.3–fold and 4.5–fold, respectively, enriched in the plasma membrane fraction as compared to crude homogenates. Protein was determined by the method of Lowry *et al.* [22] with BSA as a standard.

Fluorescence measurements Aliquots of OB-PMV were suspended in PBS at a protein concentration of 50 μg/m*l*. The incorporation of Py-3-Py was started by adding aliquots of a stock solution of 5×10^{-5} M in ethanol to membrane suspension, so that the final probe concentration was less than 5×10^{-7} M. The mixture was incubated at 4°C for 12 hr under gentle stirring. The fluorescent probe DPH was incorporated into membrane suspension at a concentration of 0.5 μg/m*l*. DPH was dissolved in tetrahydrofuran, and a volume of 0.5 μ*l* of tetrahydrofuran per ml of PBS was added directly to the membrane suspension. The mixture was incubated at 37°C for 30 min in the dark. After incorporation of the probes, the membrane suspension was placed in cuvettes. Control levels of fluorescence were then determined, different doses of rPTH-(1-34) were added directly to the cuvette, and fluorescence was again determined 30 min after addition of rPTH-(1-34). The excitation wavelength of Py-3-Py was 330 nm, and the excimer to monomer fluorescence intensity ratio (I′/I) value was calculated from the 480 to 379 nm signal ratio. The excitation wavelength of DPH was 362 nm while emission was observed at 424 nm. Fluorescence measurements were carried out at 37°C using an Aminco SLM AB2 spectrofluorometer (Spectronic Instruments, Inc., Rochester, NY, USA).

The intensity of the components of the fluorescence that were parallel (I_{VV}) and perpendicular (I_{VH}) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers oriented vertically and horizontally. The anisotropy is given by

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{1}$$

where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical (I_{HV}) to horizontal (I_{HH}) components when the exciting light is polarized in the horizontal direction. The limiting anisotropy of DPH was determined directly from the anisotropy value using the following relationship [23]:

$$r_\infty = (4/3)r - 0.10 \qquad 0.13 < r < 0.28 \tag{2}$$

The limiting anisotropy reflects restriction to probe motion and can be converted to an order parameter [24],

$$S = \sqrt{(r_\infty / r_0)} \tag{3}$$

where r_0, the anisotropy in the absence of motion, is equal to 0.362 for DPH [25].

Determination of individual monolayer structure in OB-PMV: Selective quenching of DPH This experimental determination

of individual monolayer structure in OB-PMV is based on a method previously established for LM plasma membranes [26] and synaptic plasma membranes [27,28]. This method does not simply provide a theoretically calculated or average value but is based on the assumption that the system is composed of fluorescing compartments of different accessibility to TNBS. If the fluorescence intensity F and anisotropy r are measured simultaneously, then

$$r = \sum F_j r_j \tag{4}$$

where F_j is the fraction of fluorescence intensity in compartment j. For a binary system composed of the outer and inner monolayer of the OB-PMV, this leads to

$$r = \frac{F_i}{F} r_i + \frac{F - F_i}{F} r_o \tag{5}$$

where F and F_i are fluorescence of DPH obtained for OB-PMV isolated from cells incubated with buffer A and buffer A plus TNBS at 4°C (nonpenetrating conditions), respectively.

The value of the fluorophore concentration-independent parameter anisotropies r_o (bulk anisotropy) and r_i (inner monolayer anisotropy) were determined for DPH in OB-PMV obtained from cells incubated with buffer A and buffer A plus TNBS at 4°C, respectively. Equation was then solved for r_o (outer monolayer anisotropy). Similar calculations were performed by simultaneous measurement of fluorescence intensity and either limiting anisotropy or order parameter.

RESULTS AND DISCUSSION

Given the structural complexity of the bilayer lipids, it is useful to characterize the fluidity of a given membrane in terms of several modes of motion. The excimer fluorescence and fluorescence polarization reflect the lateral and rotational diffusion, respectively, of the fluorophores. The estimation of intramolecular excimer fluorescence intensity of Py-3-Py and steady-state fluorescence anisotropy of DPH is particularly simple and trustworthy for the assessment of membrane fluidity. So, it has been employed to monitor the fluidity changes in a number of native and model membranes [13,14,17,29-32]. The chemical structures of Py-3-Py and DPH are shown in Figure 1.

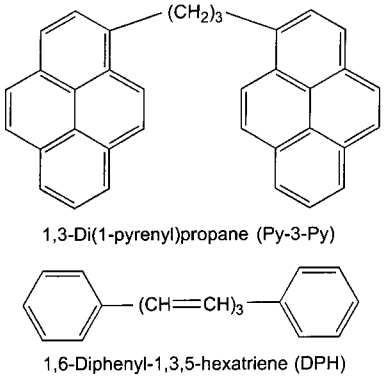

Figure 1. The chemical structures of 1,3-di(1-pyrenyl)propane (Py-3-Py) and 1,6-diphenyl-1,3,5-hexatriene (DPH).

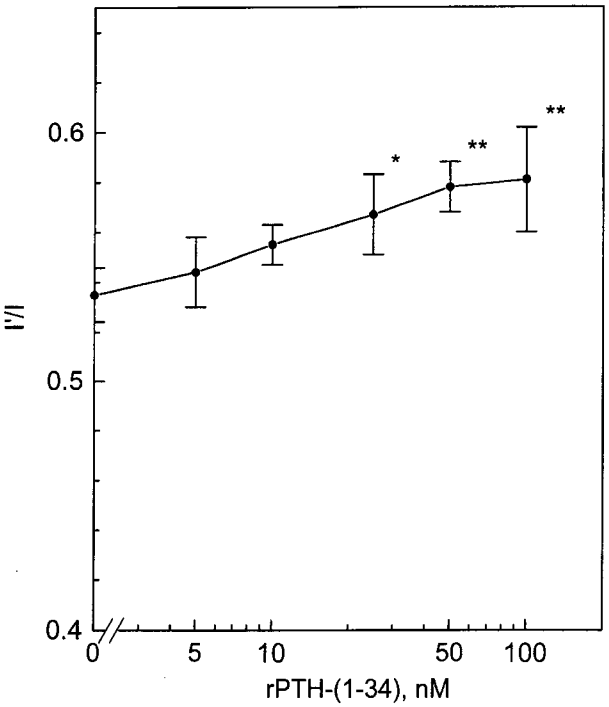

Figure 2. Effects of rPTH-(1-34) on the excimer to monomer fluorescence intensity ratio, I′/I, of Py-3-Py in the plasma membrane vesicles isolated from cultured osteoblasts. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean± SEM of 5 determinations.
*P<0.05, **P<0.01 vs. control (no PTH treatment).

At 37°C, the I′/I value of Py-3-Py in OB-PMV was 0.535± 0.011. Figure 2 shows the effects of rPTH-(1-34) on the I′/I values of Py-3-Py in OB-PMV. rPTH-(1-34) increased the I′/I ratio of the probe molecule, i.e., the lateral mobility of OB-PMV in a concentration-dependent manner. A significant increase in I′/I was observed above 25 nM rPTH-(1-34).

Selective quenching of DPH fluorescence by trinitrophenyl groups was utilized to examine the transbilayer fluidity asymmetry of OB-PMV. The validity of this method has been well demonstrated in a variety of the plasma membrane vesicles isolated from cultured cells [26-28,30-32]. The absorption peak of the trinitrophenyl groups covalently linked to outer monolayer amino groups has a large overlap with the fluorescence emission of DPH. This spectral overlap of donor emission and acceptor absorbance is responsible in part for the high resonance energy transfer efficiency of the probe. The values of fluorescence parameters in intact OB-PMV (both monolayers) as compared to those for TNBS-treated OB-PMV (inner monolayer) are listed in Table 1. The anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) of DPH in the inner monolayer were 0.024, 0.032, and 0.062, respectively, greater than calculated

Table 1. Asymmetry of DPH motion in the plasma membrane vesicles isolated from cultured osteoblasts

Membrane	Anisotropy (r)	Limiting Anisotropy (r_∞)	Order Parameter (S)
Inner+Outer	0.194±0.003	0.159±0.004	0.664±0.009
Inner	0.207±0.004	0.175±0.005	0.695±0.013
Outer	0.183±0.005**	0.144±0.007**	0.632±0.016**

Cells were treated±2 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS), pH 8.5, at 4°C for 40 min, and the plasma membrane vesicles were isolated. DPH was incorporated, and fluorescence measurements were performed at 37°C. Values from untreated membranes represent inner+outer monolayers; values from TNBS-treated membranes represent the inner monolayer; and values for the outer monolayer were calculated using Eq. (5). Values are represented as the mean±SEM of 4 determinations.
**P<0.01 vs. inner monolayer.

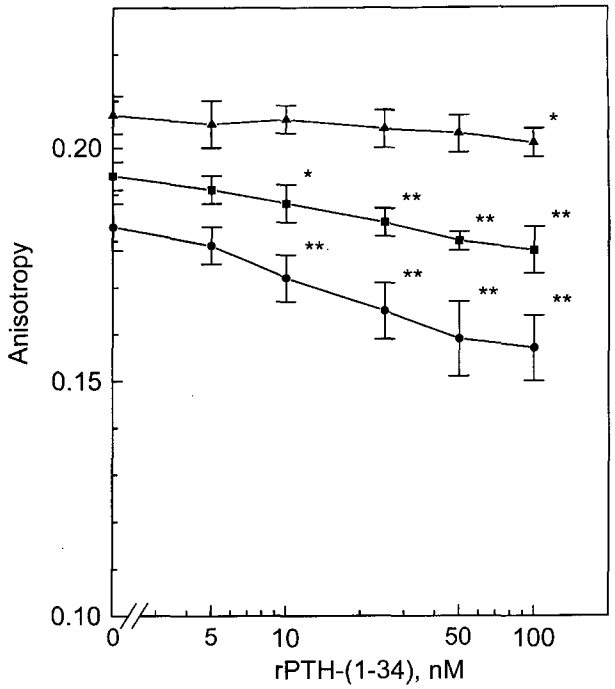

Figure 3. rPTH-(1-34) alters the anisotropy (r) of DPH in outer monolayer of the plasma membrane vesicles isolated from cultured osteoblasts. Cells were treated±2 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS), pH 8.5, at 4°C for 40 min, and the plasma membrane vesicles were isolated. DPH was incorporated, and fluorescence measurements were performed at 37°C. Untreated (inner and outer monolayer, ■); TNBS-treated (inner monolayer, ▲); and calculated for outer monolayer (●) using Eq. (5). Each point represents the mean±SEM of 5 determinations.
*P<0.05, **P<0.01 vs. control (no PTH treatment).

for the outer monolayer of OB-PMV. Since a decrease of the anisotropy of DPH reflects an increase of the rotational mobility, this result suggests that the outer monolayer of OB-PMV shows a higher degree of rotational mobility. This may be due to the asymmetric distribution of cholesterol, phospholipids, fatty acyl groups, etc., within the transbilayer domains of the OB-PMV. Transbilayer phospholipid asymmetry in OB-PMV was inferred by Libera *et al.* [33] that the aminophospholipids phosphatidylserine and phosphatidylethanolamine are mainly concentrated in the inner monolayer and the choline-containing phospholipids phosphatidylcholine and sphingomyelin are in the outer monolayer. They observed that the analogs of the aminophospholipids moved rapidly to the inner monolayer, where the choline-containing analogs disappeared more slowly from the outer monolayer in human osteoblast cells.

Selective quenching of DPH fluorescence by trinitrophenyl groups was also utilized to examine the transbilayer effects of PTH on the rotational mobility of OB-PMV. The effects of increasing concentrations of rPTH-(1-34) on the anisotropy of DPH in OB-PMV individual monolayers are shown in Figure 3. rPTH-(1-34) dose-dependently decreased the anisotropy of DPH in the bulk OB-PMV (Figure 3, closed squares). Even at 10 nM, a significant fluidizing effect was observed in the bulk OB-PMV. rPTH-(1-34) showed a greater fluidizing effect on the outer monolayer (Figure 3, closed circles) as compared to the inner monolayer (Figure 3, closed triangles). Thus, it is suggested that PTH exhibits a selective rather than nonselective fluidizing effect with transbilayer domains of the OB-PMV.

An important point of the present study is that the fluidizing effect of PTH was observed at the concentrations where PTH exerts its effects on cyclic AMP and intracellular Ca^{2+} level [34-36]. It is thus likely that the binding of PTH to the PTH/PTHrP receptor may induce conformational changes of the receptor, which can be transferred to the surrounding lipids, resulting in a change of membrane fluidity. Alterations in the fluidity of the OB-PMV may in turn influence the activity of the PTH/PTHrP receptor and/or membrane-bound enzymes through a change in the conformational flexibility. The observation that PTH exerted a greater fluidizing effect on the outer monolayer of OB-PMV raises the possibility that the hormone-receptor interaction may induce fluidity changes in some lipid domains of the membrane, while others are apparently little or not affected. There is increasing evidence for domain structures in membranes, which differ in sizes and shapes [37-40]. Thus, the present results might underestimate the effects of PTH on the fluidity of specific membrane domains because only bulk lateral mobility and bulk and transbilayer rotational mobility of OB-PMV were determined. Further experimentation is required to determine the membrane actions of PTH on specific membrane domains and to be able to define a relationship between the biological activity of PTH and changes in membrane fluidity.

In conclusion, the results of the present study indicate that, although it is obvious that PTH exerts its biological actions mainly by binding to the PTH/PTHrP receptor and downstream signaling pathways, changes in membrane fluidity may be involved in PTH action as well. To my knowledge, the results presented herein are the first to demonstrate the membrane actions of PTH and the transbilayer fluidity asymmetry of OB-PMV.

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