Parathyroid hormone transport effects and hormonal processing in primary cultured rat proximal tubular cells

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The development of satisfactory cell culture models for the study of parathyroid hormone (PTH)-induced inhibition of P_i transport has proven difficult. Using subcellular fractionation techniques we investigated the response of primary cultures of rat proximal tubular cells to PTH-(1–34). Specific binding of ^{125}I-bPTH-(1–34) occurred at 2 °C. After 5 min of rewarming, trypsin-releasable radioactivity decreased from 90 to 50%, indicating internalization of the ligand. Cell disruption, followed by density centrifugation with 17% Percoll either directly after binding at 2 °C or post-rewarming for 20 min, showed a shift of ^{125}I label from the plasma membrane (5′-nucleotidase) to lysosomal fractions (β-D-glucosaminidase), confirming the sequential occurrence of cell surface binding, internalization and transport to lysosomes of ^{125}I-bPTH-(1–34). Reculture at 37 °C revealed steady accumulation of trichloroacetic acid-soluble radioactivity in the medium, indicating degradation of ^{125}I-bPTH-(1–34). Phosphate transport in the absence of sodium was minimal. Incubation of the cells with bPTH-(1–34) resulted in up to 50% inhibition of sodium-dependent phosphate transport. Prior phosphate depletion abrogated the response to PTH.

INTRODUCTION

Since 1976, several continuous kidney proximal cell lines have become available, and significant progress has been made in elucidating the responses to parathyroid hormone (PTH) in these cells [1–5]. However, the findings in relatively dedifferentiated cell lines may not necessarily apply to the situation *in vivo* [6]. Moreover, the discrepancy between the short-lived time course of PTH second messenger responses (adenylate cyclase and phospholipase C) and the prolonged inhibition of P_i transport has not been explained [7,8]. It would be advantageous to carry out studies in primary cultured rat proximal tubular cells which should retain better differentiated functions [6]. Furthermore, no observations have been reported utilizing cell fractionation techniques in the investigation of the temporal discrepancy between second messenger activation and P_i transport inhibition, which may be a function of PTH receptor processing. To this end, we have successfully grown rat proximal tubular cells in primary culture [9], and in this paper we present further functional characterization of the subcellular components. We also report evidence of specific binding, internalization, transport to lysosomes and degradation of radioiodinated bovine PTH-(1–34) {^{125}I-bPTH-(1–34)}, with dose-dependent inhibition of P_i transport. The data indicate that the cells retain many significant proximal tubular characteristics and provide new insights into the processing of PTH-(1–34) by the proximal tubule.

EXPERIMENTAL

Materials

Chemicals were obtained from Fisher Scientific, Pittsburgh, PA, U.S.A. (chloroform, acetonitrile, trifluoroacetic acid, Hepes and sucrose) and Sigma, St. Louis, MO, U.S.A. (trypsin inhibitor, collagenase, Percoll, cortisol, insulin, transferrin, prostaglandin E_1, trypsin, fetal calf serum and EDTA).

Statistics

Student's *t* test for unpaired variables was used for statistical analysis.

Cell culture

Proximal tubular cells were prepared from rat kidney cortex by the method of Vinay et al. [9]. The tubules were cultured in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F_{12} medium supplemented with insulin, transferrin, epidermal growth factor, cortisol and prostaglandin E_1. After 5–7 days, near-confluent monolayers were obtained. These cells retain good proximal tubular characteristics, as indicated by increased cyclic AMP production in response to PTH. The cells also retain significant levels of 25-hydroxyvitamin D_3 1α-hydroxylase, alkaline phosphatase and δ-glutamyltranspeptidase and show Na^+-dependent glucose and P_i transport. The methodology and characteristics have been described in detail [10].

Iodination and purification of PTH

bPTH-(1–34) was iodinated using the Iodogen reagent and purified by h.p.l.c. [11].

Binding studies

^{125}I-bPTH-(1–34) was thawed and dissolved in 0.5 mM Hepes and 5% BSA. The solution contained 150000–200000 c.p.m. of radioactivity/ml, with or without appropriate concentrations of non-radioactive peptide, in a final volume of 1 ml. All incubations were carried out on ice in a cold room (0–4 °C). The confluent dishes were washed twice with PBS and chilled for 20 min. Ligand was then added and binding allowed to continue for various time periods. The solution was removed and the cells were washed five times with BSA/Hepes and twice with PBS.

Abbreviations used: (b)PTH, (bovine) parathyroid hormone; DMEM, Dulbecco's modified Eagle's medium; PNS, post-nuclear supernatant; NP, nuclear pellet.

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The cells were dissolved in 1% SDS and radioactivity was counted in a Beckman scintillation counter; radioactivity is expressed as c.p.m./mg of cell protein.

Protein estimations

Protein estimations were performed according to the method of Bradford [12] or Lowry et al. [13].

Internalization of PTH

After binding of ^{125}I-PTH at 2 °C, the cells were washed as before and recultured at 37 °C for 0, 2, 5, 15 or 30 min in DMEM/F_{12}. The cells were then chilled and incubated for 1 h at 2 °C in 1% trypsin. At 1 h the detached cells were centrifuged. The supernatant was separated, the cells were dissolved in 1% SDS and both pellet and supernatant were counted. The radioactivity in the supernatant (i.e. proteinase-digestible) was expressed as a percentage of total radioactivity, i.e. that in the supernatant plus cell-associated radioactivity (proteinase-inaccessible and therefore internalized).

Cell fractionation

Following finding of ^{125}I-bPTH at 2 °C, the cells were washed with PBS and then scraped in 0.5 ml of 0.25 M sucrose into a 7 ml Dounce homogenizer (Fisher). For some experiments, reculturing at 37 °C in DMEM/F_{12} was carried out before scraping. Between four and six sets of 15 strokes, separated by centrifugation at 850 *g* for 10 min in a TJ6 refrigerated centrifuge (Beckman, Irvine, CA, U.S.A.), allowed collection of post-nuclear supernatant (PNS) and nuclear pellet (NP) fractions. Phase-contrast microscopy (Olympus, Lake Success, NY, U.S.A.; model 206560) ensured that cell breakage was essentially complete and that nuclear disruption was minimal. The NP was resuspended in 0.5 ml of 0.25 M sucrose, while 0.5 ml of the PNS was taken for analysis. The remaining 4.5 ml of the PNS was loaded on to a 17% Percoll solution containing 0.25 M sucrose, 0.1 mM EDTA and 5 mM Hepes, and ultracentrifuged for 30 min at 100000 *g* (Beckman LS 7500 ultracentrifuge) using a VTI 50 vertical rotor. The resulting sigmoidal continuous density gradient (over a 3 ml cushion of 2.5 M sucrose) was collected from the top in 1 ml fractions. Each fraction was assayed for 5′-nucleotidase [14] (a marker for plasma membranes), β-D-glucosaminidase (hexase; a lysosomal marker) [15] and radioactivity. Correlation of the enzyme activity and the radioactivity of the density-gradient fractions with the original activities of the NP and PNS permitted assessment of the efficiency of the cell fractionation procedure and of the recovery of activity from the PNS.

Degradation of PTH

Following binding of ^{125}I-bPTH at 4 °C, the cells were washed and reincubated in DMEM/F_{12} medium at 37 °C for increasing periods of time. At the end of the incubation the medium was subjected to 10% trichloroacetic acid precipitation to assess the fraction of the total (supernatant plus cell-associated) radioactivity that represented trichloroacetic acid-soluble radioactivity in the medium.

Phosphate transport

Regular DMEM/F_{12} was replaced with DMEM/F_{12} containing 0.1 μM PTH and 0.5% BSA and incubations were carried out for 3 h at 37 °C. The medium was removed and replaced with working solution (152 mM NaCl or 152 mM choline chloride, 2.5 mM $NaHCO_3$, 3.5 mM KCl, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 2.5 mM glutamine and 10 mM mannose, adjusted to pH 7.5), i.e. phosphate-free solution with or without sodium. Phosphate uptake was then initiated by adding 10 μl of 0.1 mM K_2HPO_4 containing ^{32}P and the reaction was allowed to proceed for 10 min at room temperature. The working solution was removed and ice-cold stopping solution was added (100 mM NaCl, 1 mM Tris and 1 mM sodium arsenate, pH 7.5). The cells were then washed and solubilized in 0.2 M NaOH for 30 min at 37 °C. Portions of cell solution were taken for scintillation counting and protein estimation. Results are expressed as pmol of phosphate uptake/min per mg of cell protein.

RESULTS

Binding of PTH

Binding of ^{125}I-bPTH-(1–34) at 0 °C represented 10% of the total radioactivity added, was time-dependent, reached equilibrium in 30 min and was 50% inhibitable by 1 μM non-radioactive PTH. Inhibition of binding was consistent; Figure 1 represents a typical experiment.

Internalization of PTH

Cell-surface-bound ligand is accessible to exogenous trypsin, while internalized ligand is not. At time zero, before reincubation, 90% of the cell-associated radioactivity was trypsin-degradable. After rewarming for 2 min only 80% of the radioactivity was released by trypsin, and by 5 min 50% of the total was trypsin-inaccessible and thus had been internalized. Figure 2 illustrates a representative experiment. In separate experiments (results not shown), 90% of the ^{125}I-bPTH bound non-specifically in the presence of 1 μM non-radioactive bPTH was released by trypsin, even after rewarming. This indicates that only the specifically bound ^{125}I-bPTH was internalized by the cells.

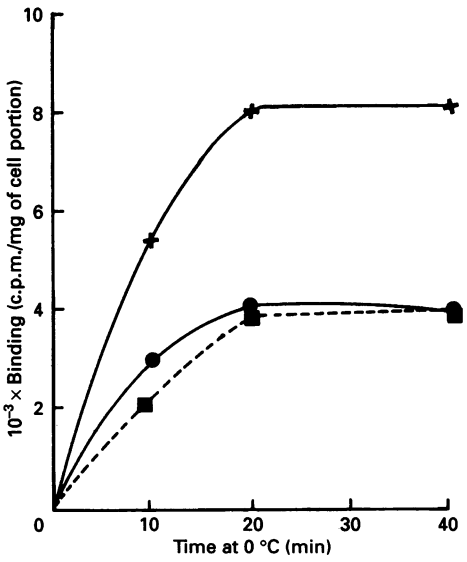

Figure 1 Time course of binding of ^{125}I-bPTH-(1–34)

Binding was measured in the absence (+) and presence (●) of 1 μM non-radioactive bPTH-(1–34). ■, Specific binding.

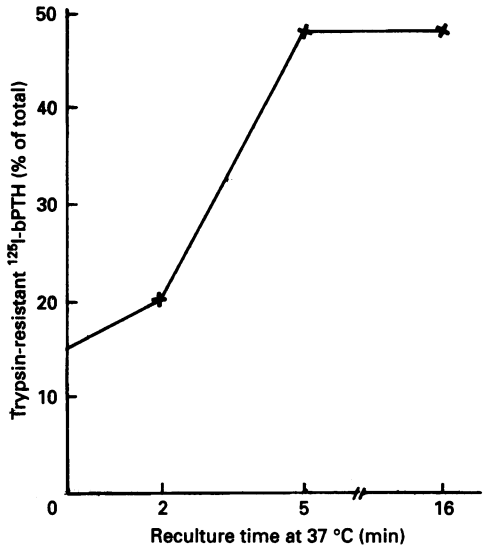

Figure 2 Measurement of trypsin-inaccessible ^{125}I-bPTH-(1–34)

Time zero is immediately after cell binding at 0 °C; '16' represents 16 min of rewarming at 37 °C. ^{125}I-bPTH-(1–34) became trypsin-inaccessible during reculture at 37 °C, indicating internalization of the ligand.

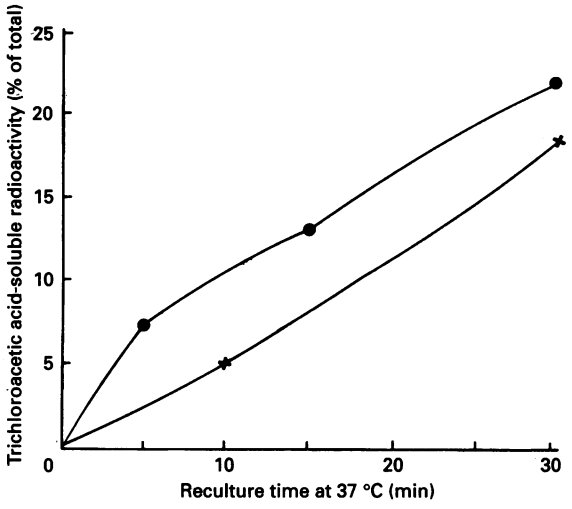

Figure 3 Degradation of ^{125}I-bPTH-(1–34)

Trichloroacetic acid-soluble radioactivity was measured in the medium during reculture at 37 °C. Two separate experiments are shown.

Degradation of PTH

Intact bPTH-(1–34) is essentially 100% insoluble in 10% trichloroacetic acid (results not shown). After 5 min of rewarming at 37 °C, 5% of the total radioactivity was recovered in acid-soluble form from the medium. At 15 min the acid-soluble fraction represented 15% of the total and at 30 min this had increased to 20%. This accumulation of acid-soluble radioactivity in the medium implies release of protein-free ^{125}I-tyrosine or acid-soluble oligopeptides into the medium from the cells, and thus the degradation of ^{125}I-bPTH (Figure 3).

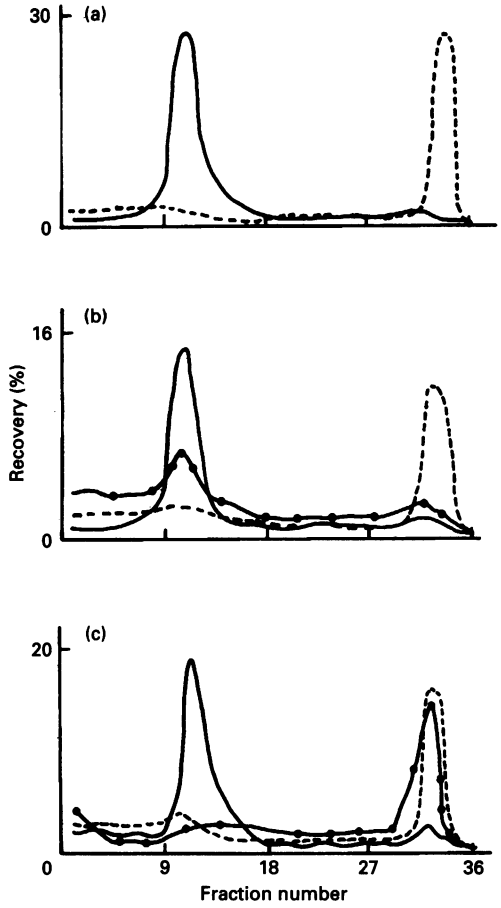

Figure 4 Percoll density gradient analysis

(**a**) Separation of plasma membrane (nucleotidase; ——) from lysosomes (β-D-glucosaminidase; ------). (**b**) Peak of ^{125}I activity (●) cosedimenting with nucleotidase after binding at 0 °C. (**c**) The ^{125}I peak has shifted to the hexase peak after rewarming to 37 °C for 20 min, indicating transport to the lysosomes.

Cell fractionation

Using the techniques described, less than 10% of the total radioactivity and marker enzyme activity for the plasma membrane and lysosomes was recovered in the nuclear pellet. Separation of plasma membranes (5′-nucleotidase) from lysosomes (β-D-glucosaminidase) was complete (Figure 4a). Recovery of each marker from the gradient relative to the total in the PNS was approx. 90%. Fractionation following binding of ^{125}I-bPTH in the cold showed a peak of ^{125}I co-sedimenting with the peak of 5′-nucleotidase activity. This is consistent with cell-surface binding of the ligand, as the plasma membrane fractions are found in the less dense portion of the density gradient. The dense lysosomal fractions (glucosaminidase peak) were well separated from the plasma membrane fractions (Figure 4b). Fractionation after 20 min of rewarming showed that a peak of ^{125}I labelling was now located with the peak of glucosaminidase activity, consistent with transport to lysosomes (Figure 4c). The rapidity of this transport is consistent with cell surface receptor binding and subsequent receptor-mediated endocytosis followed by transport to lysosomes. In earlier studies we have shown rapid (10 min) transport of α-macroglobulin to lysosomes following cell surface binding in proximal tubular cells [16].

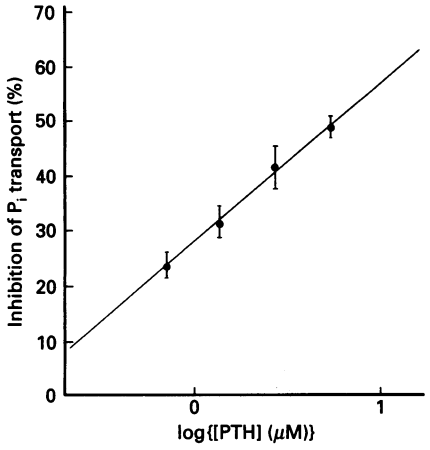

Figure 5 Inhibition of P_i transport by bPTH-(1–34)

Values are means ± S.E.M.

Phosphate transport

Phosphate transport was decreased by 96–99 % in the absence of sodium (results not shown). In the absence of PTH, phosphate transport was 570 ± 44 pmol/min per mg of protein (mean ± S.E.M.). PTH inhibited P_i transport in a dose-dependent manner (Figure 5), by up to 50 %. In contrast, vasopressin had no effect on P_i transport. Na^+-dependent glucose transport was not affected by a 3 h incubation with PTH, indicating that the effect of PTH on P_i transport was not due to generalized cell damage (results not shown).

DISCUSSION

These primary cultures of proximal tubular cells retain well-differentiated enzyme and transport functions [9], and offer an attractive model to investigate whether hormonal processing may influence the cellular response to PTH. The present studies represent further characterization of these cells and describe the methodology for successful cell fractionation. Specific binding of ^{125}I-bPTH-(1–34) was consistently shown. The binding was time-dependent, and was 50 % inhibited by 1 μM non-radioactive PTH-(1–34). The decrease in trypsin-releasable activity following rewarming was almost completely prevented by the presence of 1 μM bPTH-(1–34), indicating highly specific internalization. Cell fractionation allowed us to study two subcellular organelles, the plasma membrane and the lysosomes, and has provided more direct evidence for the internalization of [^{125}I]bPTH. The ^{125}I peak was closely related to the plasma membrane marker 5′-nucleotidase after binding at 4 °C, but migrated with the denser lysosomes following reculture at 37 °C for 20 min, a typical finding with peptide hormones. Degradation of PTH, with progressive accumulation of trichloroacetic acid-soluble activity in the culture medium, occurred during reculture at 37 °C. Density gradients also showed a decrease in the total amount of ^{125}I following reculture at 37 °C, indicating PTH degradation and loss of ^{125}I label from the cells. This coincided with an increase in the trichloroacetic acid-soluble radioactivity in the medium (results not shown). In contrast, other systems, such as opossum kidney (OK) cells, have not shown degradation of bPTH-(1–34) [5]. Renal tubular degradation of PTH-(1–34) does occur *in vivo* [17,18]. The cultures also demonstrated a response to PTH, with a highly significant inhibition of phosphate transport following incubation with PTH for 3 h. Notably, phosphate transport was 96–99 % decreased in sodium-free medium. Sodium dependency was also present in the younger cultures (only 20–30 % confluent), in contrast to the situation reported in OK cells, in which maximal sodium dependency and PTH responsiveness is only manifested in fully confluent or superconfluent cells [19]. Thus the primary culture cells may be better differentiated. Phosphate depletion of the cells, by incubating them for 30 min in P_i-free medium, abolished any detectable response to PTH. P_i depletion also abolishes the phosphaturic response to PTH *in vivo* [20].

In summary, these primary cultures of rat proximal tubular cells retain many important functional characteristics of the proximal tubule. In this paper we have shown specific cell-surface binding of ^{125}I-bPTH-(1–34), internalization of ligand and evidence for degradation of the hormone. In addition, dose-dependent inhibition of sodium-dependent P_i transport occurred in response to PTH. The cells should prove a useful model in the further investigation of the response of proximal tubular cells to PTH and the cellular regulation of P_i transport.

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