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Effects of aluminum on the parathyroid hormone receptors of bone and kidney

K.K. PUN, P.W.M. HO, and P. LAU

Department of Medicine, University of Hong Kong, Hong Kong

Effects of aluminum on the parathyroid hormone receptors of bone and kidney. Aluminum intoxication is associated with low osseous remodeling rate and peripheral resistance to parathyroid hormone (PTH). The pathophysiological mechanism of these aluminum induced changes was investigated using cultured clonal osteoblastic UMR-106 cells as well as dog renal cortical membrane. Both systems possess high-affinity PTH receptors that are coupled to adenylate cyclase. The UMR-106 cells have typical osteoblastic features, including receptors for the tissue-specific hormones, formation and mineralization of a bone-like ground substance and exclusive synthesis of type 1 collagen. The results show that aluminum at a concentration of 4 μM and 40 μM significantly inhibits the cyclic AMP responses to PTH challenge in UMR-106 cells, and this is associated with significant decrease in the binding to the PTH receptor. At 200 μM, no PTH-responsive adenylate cyclase or binding to receptor can be demonstrated. The effect of aluminum on UMR-106 rat osteosarcoma cells is not due to changes in cell number, cell viability or rate of mitogenesis. Similar results are obtained with dog kidney membrane. At a concentration of 10 μM and 400 μM, there is significant inhibition of the binding of PTH to kidney membrane and proportional decrease in PTH-stimulated adenylate cyclase. With higher concentration of aluminum, no response or binding can be demonstrated. In conclusion, aluminum at concentrations of 4 to 400 μM is associated with a decrease in affinity of PTH receptor and concomitant suppression of PTH-stimulated adenylate cyclase. The two processes are affected to a similar degree by different concentrations of aluminum, and it is likely that the primary effect of aluminum is on the affinity of the receptor which may be related to exchange of aluminum with magnesium at G-protein. This effect could account for the observed peripheral resistance to PTH in aluminum intoxication as well as the suppression of osseous remodeling rate in these patients.

Aluminum intoxication is an important cause of morbidity and mortality in hemodialysis patients [1]. Aluminum accumulation in uremic patients is responsible for the development of vitamin D resistant osteomalacia [2, 3], microcytic anemia [4], dialysis encephalopathy [5], and generalized non-specific ill-health [6]. The major source of aluminum is from the water used for preparing dialysis fluid [7]. Aluminum toxicity also occurred as a result of absorption from aluminum containing medications [8]. The aluminum toxicity syndrome can be minimized if special precautions in excluding aluminum from dialysis fluids and medications are followed. Aluminum-related osteomalacia differs from classical vitamin D-deficiency osteomalacia in that patients have an increased incidence of bone fractures, are resistant to treatment with even large doses of vitamin D and are seldom associated with secondary hyperparathyroidism [1]. Aluminum appears to affect bone formation by a direct action on osteoblasts as well as through an action at the mineralization front [9]. In addition, aluminum accumulation is associated with reduced PTH secretion, which would also reduce osteoblastic activity [10]. However, it is clear that the decrease in osteoblastic activity cannot be explained by changes in PTH levels alone [9]. It has been shown by Galceran et al with isolated perfused tibia that bones of aluminum treated dogs are resistant to the effects of PTH [11]. It has also been shown by Lieberherr et al that aluminum modulates the cyclic AMP responses to PTH in a mixed culture of mouse osteoblast-like and osteoclast-like cells [12]. To our knowledge, the possibility that aluminum may have direct effects on the function and structure of PTH receptors has not been evaluated. In this study, we investigate the effects of aluminum on the function of the parathyroid hormone receptors on osteoblasts, UMR-106 rat osteosarcoma cells, and renal tubular membrane. UMR-106 rat osteosarcoma cell is a cloned and homogeneous cell line that is known to possess typical osteoblastic features, including parathyroid hormone [13, 14] and prostaglandin-responsive adenylate cyclase [15], ability to form and mineralize a bone-like ground substance [16–18], and receptors for the tissue-specific hormones [14]. Our results show that aluminum significantly inhibits the function of parathyroid hormone receptor on kidney and bone cells.

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Methods

Materials

Synthetic bovine PTH (1-34) was prepared by Bachem Fine Chemicals (Torrance, California, USA) by the solid phase procedure and had a potency exceeding 4000 U/mg. The minimal essential medium used for culture of bone cells was obtained from the Grand Island Biological Co. (Gibco, Grand Island, New York, USA). Ten percent fetal calf serum (Gibco) as well as penicillin (100 U/ml) and streptomycin (100 μg/ml, Gibco) were added into the medium. Tissue culture dishes and plates were obtained from the Costar Laboratories (Van Nuys, California, USA). All other chemicals are from standard supplies. To measure the effects of various concentrations of aluminum ion, aluminum chloride (BDH, Poole, Dorset, UK) was included in the incubation mixture.

Cell culture

UMR-106 rat osteosarcoma cells were grown in 25 cm^2 tissue culture flask and maintained in our laboratory as described previously [19, 20]. The cells were grown on 25 cm^2 plastic flasks in minimal essential medium, supplemented with 10% fetal calf serum, 2 mM glutamine and 100 U/ml penicillin and streptomycin. Aluminum at different concentrations were included in serum-containing media during the last 24 hours of culture to evaluate their effects.

Measurement of cyclic AMP responses to PTH challenge in UMR-106 clonal rat osteoblastic cells.

Confluent cultures of UMR-106 rat osteosarcoma cells in 24-well culture plates were transferred to serum-free minimal essential medium 24 hours before use. Incubations were initiated by the addition of 150 μl of minimal essential medium containing 20 mM HEPES, pH 7.4, 1 mM isobutyl-methylxanthine, and 10 μl samples of bPTH (1-34) standards in 10 mM acetic acid with 0.1% bovine serum albumin. Incubation was carried out at room temperature for 10 minutes and was terminated by aspirating the medium, washing the cells with 1 ml phosphate-buffered saline and extracting cellular cyclic AMP with three 1 ml washes of 100% ethanol. Ethanol was subsequently evaporated in air and the extracts redissolved in 0.5 ml 50 mM sodium acetate for assay. Cyclic AMP was determined with the radioimmunoassay kit for cyclic AMP from INCSTAR Corporation (Stillwater, Minnesota, USA).

Preparation of [^{125}I][^{36}Tyr]PLP(1-36) amide

PLP, a PTH-like peptide, is a polypeptide recently sequenced by three different groups and as been shown to be of importance in the pathogenesis of hypercalcemia of malignancy [21–23]. It has been shown recently that iodinated PLP binds to the PTH receptors on bone cells and thus it is a suitable ligand for the study of PTH receptor [24].

[^{36}Tyr]PLP(1-36) amide was iodinated by a modification of the chloramine T procedure of Hunter and Greenwood [25]. It was from R.A. Nissenson of the University of California, San Francisco, USA [24]. Briefly, 10 μg of PLP peptide was added to 2 mCi of ^{125}I Na in 0.1 ml of 0.05 M sodium phosphate, pH 7.5. Iodination was allowed to proceed for three minutes following the addition of 0.6 μg of chloramine T. Fresh chloramine T was added every 60 seconds. The reaction was terminated by the addition of 20 μl of 1.0 mM β-mercaptoethanol, 20 μl of 11 mg/ml N-acetyltyrosine, and 200 μl of 10 mg/ml potassium iodide in phosphate buffer. The specific activity of the PLP peptides was about 100 μCi/μg. ^{125}I-labeled PLP peptides were purified by chromatography over Sephadex LH-20 followed by reverse-phase HPLC as previously described [24, 26].

Isolation of plasma membrane fraction from canine kidney

Fresh canine kidney tissue was obtained and processed immediately after nephrectomy as previously described [26]. The kidney specimens were sliced longitudinally and the cortical tissues were dissected away from capsular tissue and fat. The pieces of cortical tissues were homogenized with Polytron homogenizer and Teflon pestle. The homogenate was centrifuged at 2,000 g for 20 minutes and, after resuspension, was recentrifuged at 14,400 g for 20 minutes. Finally the sedimented portion was recentrifuged at 22,000 g for 15 minutes. This sedimented the plasma membranes as the top fluffy layer and the mitochondrial membrane as the bottom compact layer. The plasma membrane fraction was washed and resuspended there times before storing at −80°C. During experiments, different concentrations of aluminum were included in order to evaluate their effects on the PTH receptor.

Measurement of PTH-stimulated adenylate cyclase activity in dog kidney membrane

The PTH-stimulated adenylate cyclase activity in dog kidney membrane was measured with the method previously described [27]. The activity was measured by the conversion of [^{32}P]ATP to [^{32}P]cAMP. Incubations were carried out in a final volume of 0.1 ml containing 0.1% BSA, 20 mM MgC_{12}, 25 mM Tris-HCl (pH 7.5), 0.1 mM [^{32}P]ATP (100 to 300 cpm/pmol) and an ATP regenerating system containing 30 μg creatine phosphokinase and 12 mM creatine phosphate.

Incubations were initiated by adding plasma membrane (25 μg protein) in 25 μl SET buffer or buffer alone for blank assay measurement. After 30 minutes of incubation at 30°C, the reaction was terminated by the addition of 100 μl solution containing 50 mM Tris-HCl (pH 7.5), 2% SDS, 10 mM unlabeled ATP and 1 mM [^{3}H]cAMP (50,000 cpm).

The tubes were immediately placed in a boiling water bath for three minutes to stop the reaction. The [^{32}P]cAMP was subsequently isolated by the chromatography procedure. Column effluents containing cAMP was mixed with scintillant and the radioactivity was measured using a dual isotope program. All values were corrected for the recovery of [^{3}H]cAMP, which was consistently higher than 70%.

Preparation of [^{125}I]bPTH(1-34)

bPTH(1-34) was radiolabeled using the lactoperoxidase method. Lactoperoxidase (grade B, 20.3 IU/mg, Calbiochem, La Jolla, California, USA) was used as a catalyst and a molar ratio of bPTH (1-34), Na ^{125}I (Amersham, Buckinghamshire, UK) and H_2O_2 (Fisher, Pittsburgh, Pennsylvania, USA) of 2:1:1 was used. The iodination by lactoperoxidase technique proceeds at room temperature in 0.1 M sodium phosphate buffer, pH 7.0. The reaction was stopped by adding 100 mM dithiothreitol (Sigma Chemical Co., St. Louis, Missouri, USA), and the reactants were incubated at room temperature for 60 minutes. There was approximately 60% incorporation of radioactive iodine into protein as measured by the precipitation of aliquots of the reaction mixture with 20% trichloroacetic acid.

After labeling, labeled and unlabeled hormones were then separated by chromatography techniques to achieve maximal specific activity as previously described [28]. The products of labeling were initially extracted using Sep-Pak C_{18} cartridge (Waters Scientific, Mississauga, Ontario, Canada). After loading, reactants of the iodination procedure and unused Na ^{125}I were removed by 20 ml of 0.1% trifluoroacetic acid. Elution of the labeled hormone was subsequently achieved with 3 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid.

The eluates were further purified by high performance liquid chromatography and loaded onto a C_{18} uBondapak column (Waters Scientific), pre-equilibrated with 0.1% trifluoroacetic

Table 1. Effects of aluminium on the mitogenesis of UMR-106 bone cells

Aluminium chloride	UMR-106 cells at 80% confluence mitogenesis	UMR-106 cells at 100% confluence mitogenesis
0 M	100%	100%
10^{-6} M	100.2%	99.2%
4×10^{-5} M	101.3%	98.2%
2×10^{-4} M	98.1%	99.1%
10^{-3} M	99.2%	100.2%

Different concentration of aluminium were used to incubate with UMR-106 cells and the mitogenesis was quantitated with cells at 80% and 100% confluence. The baseline value of mitogenesis (buffer only) was taken as 100%.

acid in water. The peptides were subsequently eluted with 0 to 95% gradient of N-propanol in 0.1% trifluoroacetic acid.

Measurement of binding of ^{125}I bPTH (1-34) to dog kidney membrane

Dog kidney membrane in 10 μl ice cold SET (0.25 M sucrose, 1 mM EDTA, 5 mM Tris, pH 7.5) buffer was added to an incubation mixture containing a final concentration of 50 mM Tris, 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 0.1% BSA, 10,000 cpm [^{125}I]-bPTH (1-34) together with unlabeled bPTH (1-34) of appropriate concentrations to make up a final volume of 100 μl in 1.5 ml microcentrifuge tube. The mixture was incubated for one hour at 30°C. At the end of this period, the binding reaction was stopped by diluting with 1 ml ice cold SET. The tube was then centrifuged for five minutes at 4°C. After aspiration, the pellet was counted for gamma rays. The readings were compared with total count, blank (incubation mixture without membrane and unlabelled bPTH (1-34)), and non-specific binding, which was measured in the presence of 250 nM bPTH (1-34). The specific binding was the difference between total binding and non-specific binding.

To measure binding in the presence of aluminum ion, aluminum chloride (BDH) was included in the incubation mixture so as to make the final concentration of aluminum at 10 μM, 400 μM and 1000 μM, respectively.

Measurement of the effects of aluminum on the mitogenesis of UMR-106 rat osteosarcoma cells

Rat osteosarcoma UMR-106 cells were seeded in 24-well cluster plates (Costar, Van Nuys, California, USA) at a concentration of 1×10^5 cells/well. Different concentrations of aluminum (Table 1) were added and incubated for 24 hours at 37°C in 5% CO_2 and 95% air. [^{3}H]-thymidine (0.5 μCi/ml) (Amersham International, Buckinghamshire, UK) was included during the last six hours of incubation with aluminum. The reaction was terminated by aspiration of medium. The cultures were subsequently washed with ice-cold phosphate-buffered saline containing 1 mM thymidine. After washing, UMR-106 cells were trypsinized and transferred to centrifuge tubes containing equal volumes of 0.1% BSA in distilled water and precipitated by ice-cold TCA. After one-half hour at 4°C, tubes were centrifuged for 30 minutes and supernatants were discarded. The pellet was dissolved in 0.1 M sodium hydroxide. Mitogenesis as measured by [^{3}H]-thymidine incorporation was determined by scintillation counting.

Binding of [^{125}I]-PLP(1-36)-amide to UMR-106 rat osteosarcoma cells

Binding of labeled ligands to UMR-106 cells was measured on confluent cells in 24-well cluster plates, as previously described [24]. Briefly, confluent cells were incubated at 37°C in serum-free MEM-EBSS for one hour. One hundred and fifty microliters of MEM-EBSS containing 20 mM HEPES, 1×10^4 cpm of labeled PLP and unlabeled peptides, was then added. Incubations were carried out at room temperature for one hour to achieve the steady state. The medium was saved for measurement of unbound counts. Following removal of medium, the cells were washed three times with 1.0 ml of ice-cold PBS and were subsequently scraped from the dishes in 0.8 M NaOH for gamma counting.

Statistical analysis

Unless otherwise specified, the data were obtained from representative experiments which were performed three times. The data are presented as the mean ± standard error of the mean of triplicate assays for each condition in the cell population. The significance of difference between groups was determined by analysis of variance as well as by the Student's *t*-test. A *P* value of less than 0.5 was taken as significant.

Results

Effect of aluminum on the cyclic AMP responses to PTH in UMR-106 rat osteosarcoma cells

Incubation with aluminum at 4 μM, 40 μM and 200 μM was performed as described. The results are shown in Figure 1. The cyclic AMP responses to PTH challenge were significantly impaired in the presence of 4 μM and 40 μM aluminum and were nearly undemonstrable in the presence of 200 μM aluminum.

Effect of aluminum on the binding of [^{125}I]PLP(1-36) amide to UMR-106 rat osteosarcoma cells

Incubation with aluminum at 40 μM, 40 μM and 200 μM was performed as described. The results are shown in Figure 2. In the presence of 4 μM and 40 μM of aluminum, the binding of [^{125}I]PLP(1-36) amide to UMR-106 rat osteosarcoma cells was significantly impaired. In the presence of 200 μM aluminum, no binding could be demonstrated at all.

Effect of aluminum on the adenylate cyclase response of dog kidney membrane to PTH challenge

Figure 3 shows the results of the adenylate cyclase response of dog kidney membrane to PTH challenge. In the presence of aluminum at 10 μM and 400 μM, the adenylate cyclase response was significantly impaired. In the presence of 1000 μM aluminum, the adenylate cyclase response to PTH challenge was barely demonstrable.

Effect of aluminum on the binding of [^{125}I]bPTH(1-34) to dog kidney membrane

Figure 4 shows the results. In the absence of aluminum, the PTH receptor on dog kidney membrane had an affinity of 2 nM and was present at a concentration of 2.2 pmol/mg protein. In the presence of 10 μM and 400 μM aluminum, the affinity of PTH receptor decreased significantly. In the presence of 1000 μM aluminum, no binding activity could be demonstrated.

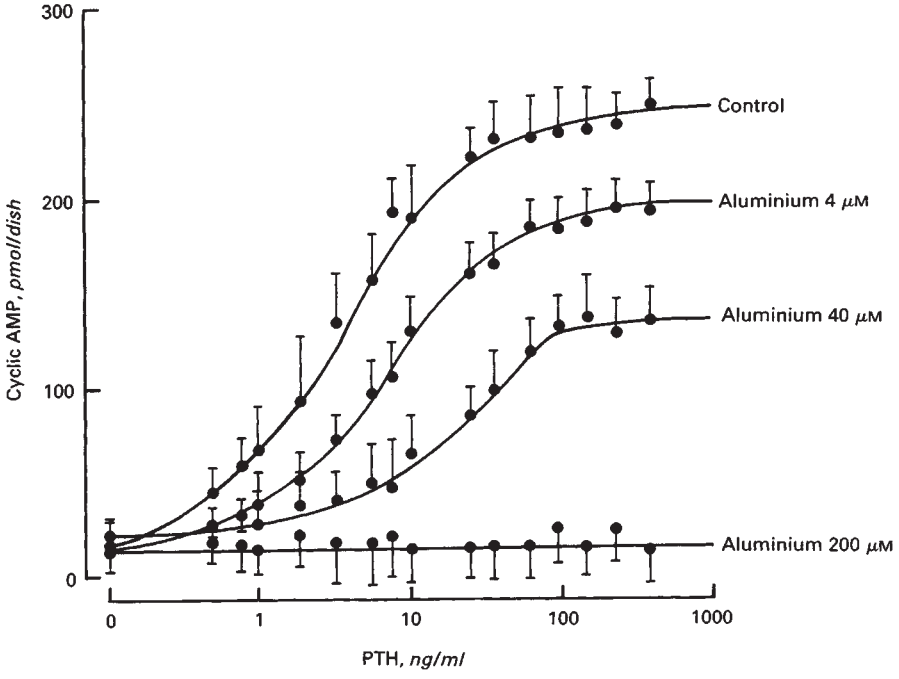

Fig. 1. *Effect of aluminum 40 μM and 200 μM on the cyclic AMP responses to parathyroid hormone stimulation in UMR-106 rat osteosarcoma cells.* Incubation with aluminum 4 μM, 40 μM and 200 μM was performed as described. The cyclic AMP responses to PTH challenge were significantly impaired in the presence of 4 μM and 40 μM aluminum ($P < 0.05$) and were undetectable in the presence of 200 μM.

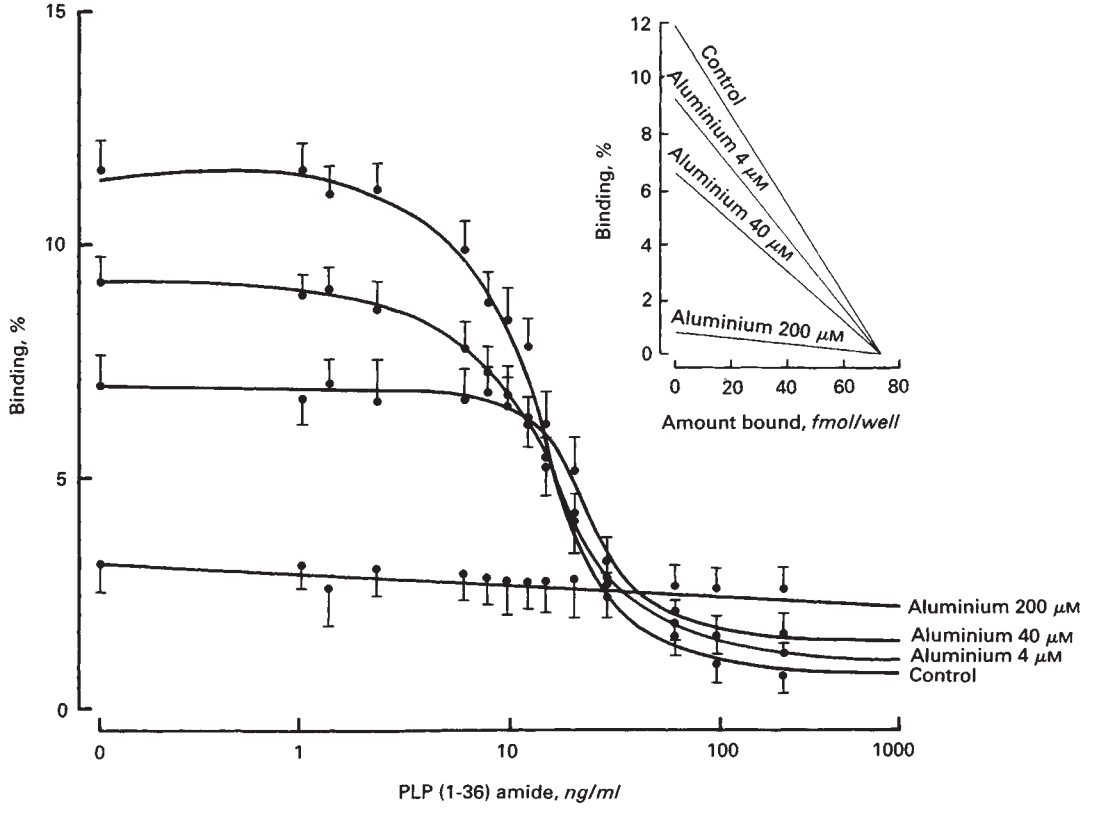

Fig. 2. *Effect of aluminum on the binding of [^{125}I]PLP(1-36) amide to UMR-106 rat osteosarcoma cells.* Similar to the results observed in Figure 1, the binding of [^{125}I]PLP(1-36) amide was significantly impaired in the presence of 4 μM and 40 μM of aluminum ($P < 0.05$), and was undetectable in the presence of 200 μM. Scatchard analysis (inset) shows that there was significant decrease in the slope (affinity of receptor) with increasing concentrations of aluminum ($P < 0.05$).

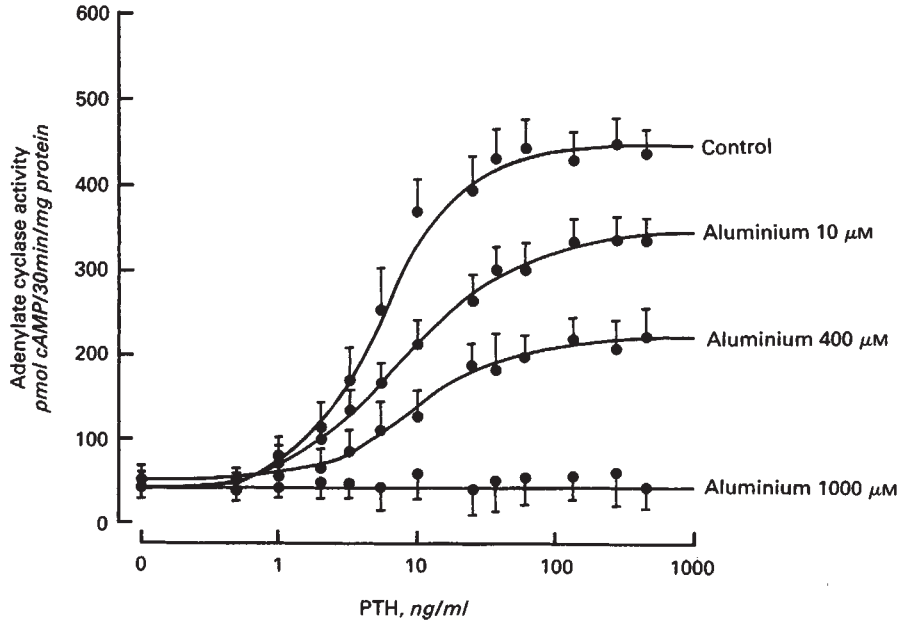

Fig. 3. *Effect of aluminum on the adenylate cyclase response of dog kidney membrane to PTH challenge.* In the presence of aluminum 10 μM and 400 μM, the adenylate cyclase response was impaired by fourfold ($P < 0.05$). In the presence of aluminum 1000 μM, the adenylate cyclase response to PTH challenge was barely detectable.

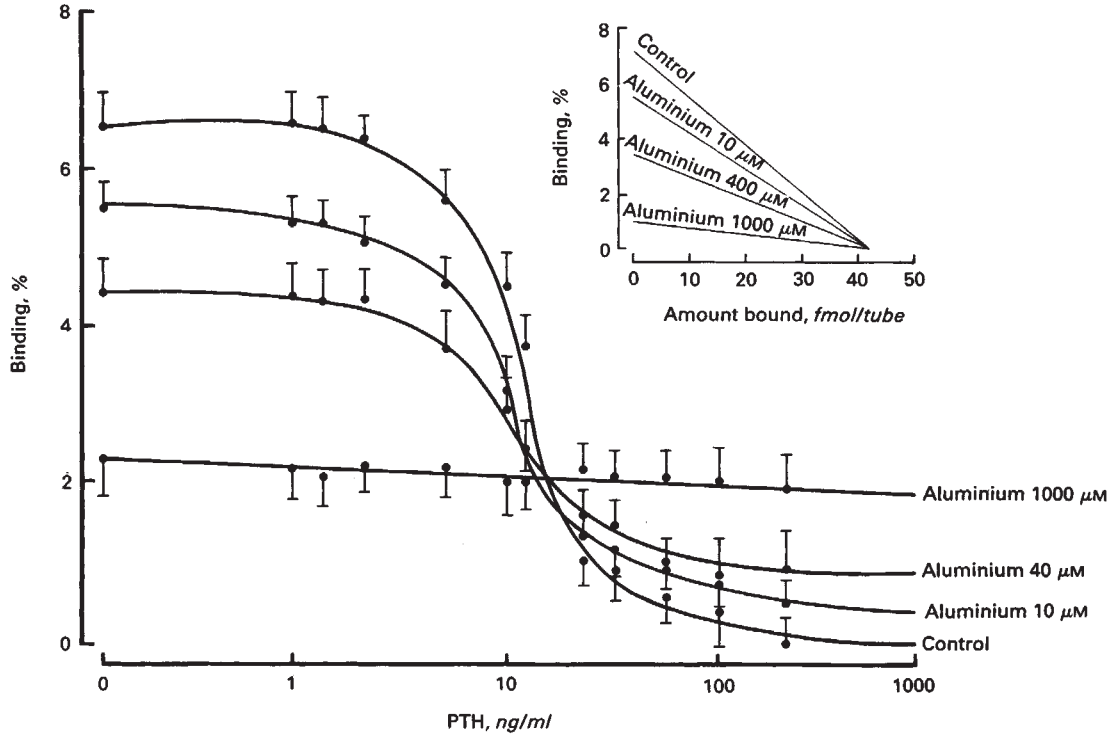

Fig. 4. *Effect of aluminum on the kidney of [^{125}I]bPTH(1-34) to dog kidney membrane.* In the presence of 10 μM and 400 μM aluminum, the affinity of PTH receptor decreased significantly ($P < 0.05$). In the presence of 1000 μM aluminum, no receptor could be demonstrated. Scatchard analysis (inset) shows that there was significant decrease in the slope (affinity of receptor) with increasing concentrations of aluminum ($P < 0.05$).

Effects of aluminum on the mitogenesis of UMR-106 rat osteosarcoma cell

Different concentrations of aluminum ions were used for incubation to assess their effects on the mitogenesis of bone cells. The results are shown in Table 1. Aluminum did not have any significant effect on the mitogenesis of bone cells when tested with cultures at two different stages of confluence. The number of viable cells as measured by trypan blue exclusion was also comparable.

Discussion

Studies on the pathogenesis of aluminum intoxication have shown that the bone aluminum content is substantially increased in uremic patients, especially among those undergoing treatment with hemodialysis [29–31]. The cellular and molecular mechanism by which aluminum affects mineralization and induces osteomalacia is unknown. It is important to define the factors which regulate the skeletal accumulation of aluminum and to identify the effects of aluminum on bone to formulate strategy for the treatment of aluminum toxicity.

Our results show that aluminum at 4 μM, 40 μM and 200 μM significantly impaired the cyclic AMP response to PTH challenge in UMR-106 rat osteoblastic cells and at 10 μM, 400 μM and 1000 μM in dog kidney membrane (Figs. 1 and 3). Because of the high protein concentration in the culture media and in the dog kidney membrane preparation, it is likely that much of aluminum is protein-bound. In association with this decrease in response, aluminum at similar concentrations significantly impaired the binding of I^{125}-PLP to PTH receptors on the UMR-106 rat osteosarcoma cells and the binding of I^{125}-bPTH (1-34) to PTH receptors on the dog renal tubular membrane. In both systems, aluminum at a lower concentration decreased the affinity of PTH receptors significantly, while aluminum at a higher concentration was associated with no demonstratable binding at all.

The I^{125}-bPTH (1-34) was prepared with lactoperoxidase method and bound with high affinity and specificity to PTH receptor on dog kidney membrane. However, it did not bind to PTH receptor on rat bone cells. This may be due to oxidative damage to PTH during iodination. Production of bioactive, radiolabeled PTH is especially difficult because of the presence of labile amino acids at bioactive sties. In this study, a similar peptide, PTH-like peptide, which has recently been cloned, bound to PTH receptor with high affinity and specificity [21–24]. Thus I^{125}-PLP(1-36) amide was used for characterization of the PTH receptors on bone cells after aluminum intoxication.

The above effects on PTH receptor are unlikely to be due to non-specific toxic effects on cells. Indeed, the observed effects of aluminum in the system of dog kidney membrane show that the toxicity acts directly on membrane proteins and cannot be explained by non-specific injury to the cells. The lack of suppressive effect of aluminum on the mitogenesis of bone cells further shows that aluminum at the concentrations used does not significantly suppress cellular function and division. Thus the observed effects of aluminum cannot be due to a decrease in cell number. Control experiments (data not shown) also show that aluminum does not have any action on insulin receptor of bone cells, and lead and copper do not have actions on PTH receptor of bone cells. These data show that the effects of aluminum on PTH receptors are specific toxic actions.

The above results demonstrate that the effect of aluminum on cyclic AMP responses to PTH is closely associated with changes in the binding affinity of the PTH receptors. The direct explanation of these conjoint changes is that aluminum interacts with the PTH-receptor adenylate-cyclase complex to decrease the binding with ligand and, as a result, the associated activation of adenylate cyclase is decreased. PTH receptor is one of the peptide hormone receptors, and the exact amino acid sequence is not yet known [32]. Like the other peptide hormone receptors, PTH-receptor adenylate-cyclase complex consists of three different components, namely receptor moiety, G-protein and adenylate cyclase [32]. The receptor moiety is known to be a polypeptide of 70 to 80 kDa in molecular weight and probably has structural homology to other peptide hormone receptors which are coupled to G-protein [33]. The G-protein has an important regulatory function. It binds guanyl nucleotides in the presence of the important cofactor, magnesium, and couples receptor occupancy to the activation of adenylate cyclase, with release of cyclic AMP into the cytoplasm [33]. The G protein is converted by GTP binding into an active form that is associated with the enzymatic catalytic unit, stimulating the conversion of ATP to cyclic AMP. Subsequently, the GTP-degrading activity associated with the G-protein causes its deactivation by converting the bound GTP to GDP and favoring its dissociation from the C unit [33]. In all G-protein-linked receptors, magnesium is an essential co-factor for its function [33]. In fact, the substrate for adenylate cyclase is Mg-ATP and not ATP per se.

Because the effective ionic radii for aluminum and magnesium are comparable, aluminum substitution for magnesium in physiological settings is a common pathogenetic mechanism for aluminum toxicity [34]. On the other hand, aluminum substitution for calcium is highly unlikely because the effective ionic volume of calcium is nine times greater than that of aluminum [34]. As magnesium has important role in the formation of the ternary PTH-receptor-Gs complex that is required for PTH-activation of adenylate cyclase, the removal of magnesium from the incubation medium used for adenylate cyclase assay would lead to loss of activity. The substitution of magnesium by aluminum would lead to similar loss of action and prolonged inactivation as the dissociation kinetics for aluminum is slow. The end results would be similar to those obtained in this study, namely loss of PTH-activated adenylate cyclase activity and loss of high-affinity binding sites for PTH. This could account for the loss of PTH-induced release of cyclic AMP for both in vivo and in vitro models [35].

To conclude, aluminum markedly suppresses the ligand binding and the action of PTH receptors in both rat bone cells and dog kidney membrane. This is the likely pathogenetic mechanism underlying the observed peripheral resistance to PTH as well as the suppression of osseous remodelling rate in aluminum intoxication.

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Reprint requests to K.K. Pun, M.D., M.R.C.P., Department of Medicine, University of Hong King, Hong Kong.

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