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Increased ATPase and Decreased Alkaline Phosphatase Activities by Parathyroid Hormone in Cultured Chick Embryo Tibiae[1] (40053)

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Bone alkaline phosphatase (AP) activity has been shown to be highest in areas of bone undergoing mineralization (1, 2), but its role and substrate(s) are not known. Magnusson *et al.* (6) performed histochemical studies which suggest that bone AP in mineralizing areas might be an alkaline ATPase that uses Ca-ATP as substrate. In contrast to this concept, Messer *et al.* (7) have shown evidence that bone nonspecific AP and ATPase might be properties of different enzymes.

The effect of parathyroid hormone (PTH) on bone AP activity has previously been studied *in vitro* with conflicting results. Compared to bones cultured without PTH, mouse calvaria incubated with the hormone have been shown to contain increased AP activity (8), decreased AP activity (9), and unchanged AP activity (10). Isolated rat bone cells incubated with PTH in the medium showed a 40% decrease in surface AP activity after only 2 hr (11). Luben *et al.* (12) have isolated a population of osteoblastlike cells from mouse calvaria. Incubation of these cells with PTH causes a substantial decrease in AP activity after 24 hr. Using embryonic chick tibiae in organ culture (13, 14), the present study was undertaken to determine and compare the effects of PTH on bone AP activity and on bone ATPase activity. Different effects of PTH on the two activities would be consistent with the concept of two separate enzymes.

Materials and methods. Organ culture. Tibiae were aseptically removed from 10- to 12-day old White Rock chick embryos and rinsed briefly in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid)-buffered salt solution, pH 7.4. The tibiae were then placed in 25 ml Erlenmeyer flasks containing 3–5 ml chemically defined culture medium. The flasks were gassed with 5% CO_2 in air, closed with silicone rubber stoppers, and placed on a rocker platform in a 38° incubator. All culture media were prepared by modification of Eagle's minimum essential medium as described by Ramp and Neuman (13). The calcium and phosphate concentrations were 1.8 m*M* and 1 m*M*, respectively. Bovine serum albumin (1 mg/ml) was added to the media to prevent the PTH from adsorbing to the glass culture flasks. Tibiae were paired between control and treatment flasks such that each embryo served as its own control. The bones were precultured for 24 hr in medium without PTH to remove extraneous AP (enzyme in blood or in the small amounts of non-bone tissue explanted with the bone). Preculture removed approximately 25% of the AP associated with noncultured bones. All of the AP activity lost from the tibiae during preculture was recovered in the medium. After preculture the medium was changed to fresh medium containing either PTH or the PTH vehicle. The incubation was then continued for 24 or 48 hr. At the end of the culture period, the bones were briefly rinsed in 0.9% NaCl solution. Bones and media were frozen and stored at −20° until use.

Unless otherwise noted, the source of PTH was Lilly Parathyroid Injection. Highly purified bovine PTH was obtained from Inolex Laboratories and the synthetic 1–34 peptide of bovine PTH from Beckman. The vehicle for Lilly Parathyroid Injection was 0.2% phenol, 1.6% glycerin, and the vehicle for the purified and synthetic PTH was 0.001 *N* HCl, 0.9% NaCl.

Analytical procedure. Bones were homogenized in distilled water with a Polytron homogenizer (Brinkmann). The homogenate and culture media were assayed for AP activ-

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ity at pH 9.8 using *p*-nitrophenylphosphate (*p*-NPP) as substrate (15). One unit of enzyme activity is defined as one micromole of substrate hydrolyzed per hr at 38°, and enzyme activity is expressed in terms of activity units (A) per bone. Bone ATPase activity was determined using the supernatant of the bone homogenate after centrifugation at 1000*g* for 10 min to remove large particles. Samples of supernatant were incubated for 1 hr with 3 m*M* ATP containing γ^{32}P-ATP (SA = 0.2 Ci/mole), 3 m*M* $MgCl_2$ in 75 m*M* Tris, pH 8.5, in a total vol of 0.2 ml. In some assays 5 m*M* levamisole (American Cyanamid Co., Princeton, NJ) was included in the hydrolysis incubation. The rate of hydrolysis of ATP in this assay was maximal at pH 8.5, proceeded at a constant rate during this time period, and was proportional to the amount of homogenate assayed. The hydrolysis was stopped with 0.2 ml 10% trichloroacetic acid. Free P_i was extracted with 0.1 ml 5% ammonium molybdate and 0.4 ml isobutanol. After mixing for 30 sec, the samples were centrifuged at 2000*g* for 10 min to separate the aqueous phase from the isobutanol (16). The free P_i in a sample of the isobutanol phase was calculated from the amount of ^{32}P as determined by liquid scintillation counting and ATPase activity is expressed as nmole of ATP hydrolyzed per bone per hr. Lactate released to the medium was measured enzymatically (17) after removal of interfering substances with Norit A charcoal. The significance levels of differences between paired control and treated bones were determined using Student's *t* test for paired data. A value of $P < 0.05$ was considered significant. Since this test of significance is based on the difference between paired bones, no inference as to the levels of significance can be made from the standard error of mean values. Therefore, these were not included in the figures.

Results. The experiment shown in Fig. 1 was performed to determine the changes in total AP activity (bone content + medium content) of tibiae during 48 hr of organ culture. Tibiae from 11-day embryos were precultured for 24 hr. One bone from each pair was then frozen, and the other was placed in fresh medium and incubated for 48 additional hr. During the 48-hr incubation, the bone content of AP activity increased 35% compared to the preculture level. When the released activity was added to the bone content, the total amount of AP activity after 48 hr of culture was 171% of the preculture activity. The most likely explanation for these data is that the tibiae were actively synthesizing AP *in vitro*. However, the possibility of activation of already synthesized enzyme cannot be ruled out.

The data in Fig. 2 show that while tibiae

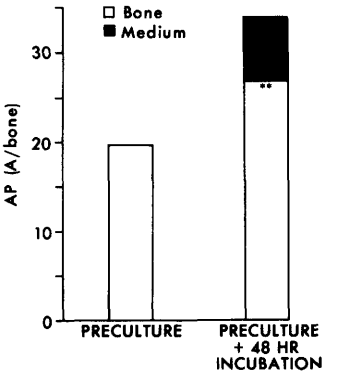

FIG. 1. Synthesis of AP by 11-day chick embryo tibiae. Paired bones were precultured for 24 hr. One bone from each pair was then frozen and the other was cultured an additional 48 hr in fresh medium. Bone homogenates and the 48 hr incubation media were assayed for AP. Each bar represents the mean value for eight tibiae. $^{**}P < 0.01$ compared to paired precultured bones.

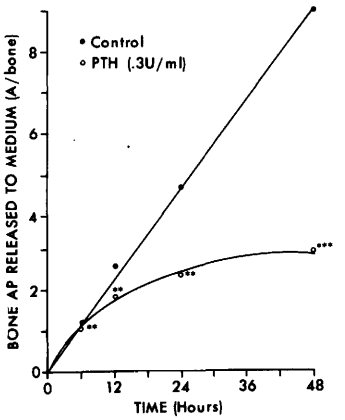

FIG. 2. Effect of PTH on the release of AP by 12-day chick embryo tibiae in organ culture. Bones were paired between media containing control and PTH (0.3 U/ml) media. Serial samples of media were collected during the 48 hr incubation (after preculture) to monitor the release of AP. Each point represents the mean value for five tibiae. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to paired control bones.

cultured in control medium released AP at a constant rate, PTH (0.3 U/ml) caused a decrease in released activity. After 6 hr the AP activity in the medium of PTH-treated bones was significantly lower than in control medium according to the paired t test, even though the difference between the means is quite small. By the end of the 48-hr culture period the PTH medium contained only 36% of the control activity. Addition of PTH to medium containing AP did not alter the AP activity, indicating that there was no direct inhibition of the AP enzyme by PTH (data not shown).

The effect of PTH to decrease the amount of AP activity released by tibiae was reflected in the bone content of AP activity. Figure 3A shows the bone content of AP after 48 hr of culture in medium containing three doses of PTH: 0.003, 0.03, and 0.3 U/ml. The decrease in bone content (Fig. 3A) and release (Fig. 3B) of AP caused by PTH is related to the concentration of PTH in the medium, with a concentration as low as 0.03 U/ml resulting in more than a 30% decrease in AP activity after 48 hr.

Three different preparations of bovine PTH (parathyroid extract, highly purified hormone, and the 1–34 peptide fragment) all caused similar decreases in AP activity. Incubation in medium containing 1 U/ml of either highly purified or synthetic hormone caused the same 60–70% reduction of both bone content and release of AP activity as observed with parathyroid extract.

Studies demonstrating that bone AP will hydrolyze ATP (18, 19) have led to the proposal that bone AP may function as an ATPase, possibly to provide energy to increase Ca efflux from bone cells (5). Because it was clear that PTH decreased the AP activity of embryonic chick tibiae (Figs. 1–3), the experiment shown in Fig. 4 was performed to determine whether the bone ATPase activity was similarly decreased. As in previous experiments, bones were precultured in control medium for 24 hr and then cultured for 48 hr in medium containing either PTH (1 U/ml) or vehicle. The ATPase assay was performed both in the presence and absence of levamisole (5 mM), an inhibitor of nonspecific bone AP (20, 21). At this concentration, levamisole caused total inhibition of the bone nonspecific AP assayed using p-NPP (data not shown). At the end of the 48-hr culture period, the total ATPase activity of PTH-treated bones was the same as that of the control bones (Fig. 4B), in contrast to the AP activity of PTH-treated bones which was again markedly decreased (Fig. 4A). When the ATPase activity due to nonspecific AP was eliminated by including levamisole in the ATPase assay,

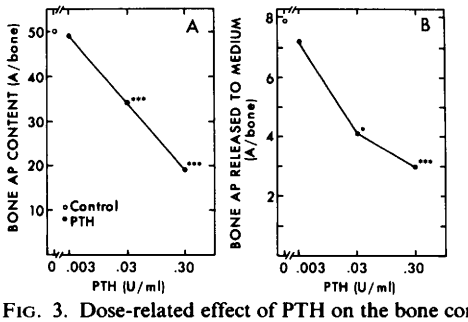

FIG. 3. Dose-related effect of PTH on the bone content (A) and release (B) of AP. Tibiae from 12-day chick embryos were paired between control medium (open circles) and PTH-containing medium (closed circles). After preculture and incubation for 48 hr, bone homogenates and culture media were assayed for AP activity. There was no significant difference in either the bone content or release of AP by control bones in different dose groups. Thus, these values were pooled and are represented by a single open circle. Each closed circle represents the mean value for five tibiae. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to paired control bones.

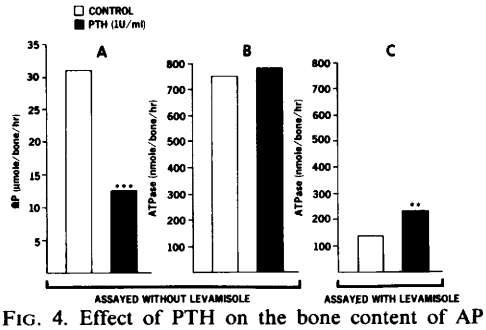

FIG. 4. Effect of PTH on the bone content of AP (A), total ATPase (B) and ATPase in the presence of 5 mM levamisole (C). Tibiae from 11-day embryos were incubated in medium containing PTH (1 U/ml) for 48 hr after a 24 hr preculture. Enzyme activity is expressed as quantity of substrate hydrolyzed per bone per hr, so that comparisons can be made between AP and ATPase activity. The mean (±SE) of the differences between the ATPase activities of paired, PTH-treated and control bones (C) is 87 ± 23. Each bar represents the mean value of 8 tibiae. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to paired control bones.

the ATPase activity of PTH-treated bones was 160% of the activity of control bones (Fig. 4C). Three additional experiments similar to the one shown in Fig. 4 have confirmed this finding. The data shown in Fig. 4 indicate that bone contains at least two phosphatase activities at alkaline pH: a nonspecific alkaline phosphatase (AP) that is decreased by PTH, and an ATPase that is increased by PTH.

Discussion. This study demonstrates for the first time, by using a selective inhibitor, the existence of an ATPase activity in bone which can be distinguished from nonspecific alkaline phosphatase. In contrast to AP activity, which was decreased in tibiae cultured in medium containing PTH, this specific ATPase was shown to be substantially increased in PTH-treated bones.

Bone AP, the enzyme inhibited by levamisole, has been associated with mineral deposition since 1923 when Robison discovered an enzyme in ossifying cartilage which would hydrolyze hexosemonophosphoric acid esters, and suggested a role for this enzyme in bone calcium phosphate deposition (1). Since that time, alkaline phosphatase has been localized in osteoblast plasma membranes and matrix vesicles in areas undergoing mineralization (2). In spite of the fact that AP occurs in mineralizing areas and seems to be necessary for the process of mineral deposition, no mechanism for this process has been clearly demonstrated. Nevertheless, the inhibition of bone AP activity by PTH suggests that PTH may exert an acute inhibitory effect on bone mineral deposition.

The present study did not determine whether the decrease in AP activity caused by PTH was due to inhibition of synthesis of new enzyme, or to the inhibition of the activity of the enzyme. Work with HeLa cell AP has suggested that cellular cAMP may be involved in regulating AP activity (22). In HeLa cells, agents leading to an increased intracellular cAMP concentration also caused decreased AP activity. It is possible that the bone cell cAMP which is increased by PTH (23) may be responsible for the decrease in AP activity.

Studies of alkaline phosphatase from rat odontoblasts involved in dentin formation have demonstrated the presence of a nonspecific AP, as well as a Ca-ATPase (21), which is active in the presence of 0.5 m*M* levamisole. Rat odontoblast AP activity was nearly completely inhibited by 0.5 m*M* levamisole, while the chick bone AP in the present study was only partially inhibited by levamisole concentrations less than 5 m*M*. The difference between the concentrations of levamisole required to inhibit mammalian bone AP and avian bone AP is consistent with a recent report by Reynolds and Dew (24) which showed that levamisole was ten times more potent as an inhibitor of mammalian bone AP than as an inhibitor of avian bone AP. As in the present study, they showed that 5 m*M* levamisole resulted in near total inhibition of avian bone AP.

It has been suggested (21) that in odontoblasts, the AP functions to remove mineralization inhibitors such as inorganic pyrophosphate and to increase the local phosphate concentration while the Ca-ATPase acts as a Ca^{2+} transporting enzyme to accumulate Ca^{2+} ions in the cells. These two odontoblast enzymes may be analogous to the two phosphatases studied in the present work. However, the increase in bone ATPase that resulted from culture from PTH suggests that the ATPase may function in PTH-stimulated Ca^{2+} efflux from bone cells rather than in accumulation of Ca^{2+} for mineral deposition. Ramp (5) has shown that an early effect of PTH is to increase the efflux of calcium from bone to medium without increasing the P_i release. It was suggested that this apparent stimulation of "Ca pumping" might be due to an increased ATPase activity. The ATPase that is not inhibited by levamisole and is increased in PTH-treated bones may be this enzyme.

Summary. Addition of PTH to the medium of embryonic chick tibiae in organ culture caused a decrease in AP activity. Total ATPase activity was the same in control and treated bones, but ATPase activity assayed in the presence of levamisole, an inhibitor of bone AP activity, was greater in PTH-treated bones. These data suggest that there are at least two different phosphatase activities in bone with optimal activity at alkaline pH: a nonspecific alkaline phosphatase which is decreased in PTH-treated bones, and an ATPase which is increased by the hormone.

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1. Robison, R., Biochem. J. **17,** 286 (1923).
2. Bernard, G. W., J. Dent. Res. **55,** B219 (1976 Abstract).
3. Fleisch, H., and Neuman, W. F., J. Amer. Chem. Soc. **82,** 3783 (1960).
4. Fleisch, H., and Russell, R. G. G., J. Dent. Res. **51,** 324 (1972).
5. Ramp, W. K. Clin. Orthop. **106,** 311 (1975).
6. Magnusson, B. C., Heyden, G., and Arwill, T., Calc. Tissue Res. **16,** 169 (1974).
7. Messer, H. H., Rogers, J., Shami, Y., and Copp, D. H., Comp. Biochem. Physiol. **51B,** 19 (1975).
8. Morgan, D. B., Monod, A., Russell, R. G. G., and Fleisch, H., Calc. Tissue Res. **13,** 287 (1973).
9. Vaes, G., J. Cell Biol. **39,** 676 (1968).
10. Messer, H. H., Armstrong, W. D., and Singer, L., Calc. Tissue Res. **13,** 227 (1973).
11. Hekkelman, J. W., and Moskalewski, S., Exp. Cell Res. **58,** 283 (1969).
12. Luben, R. A., Wong, G. A., and Cohn, D. V., Endocrinology **99,** 526 (1976).
13. Ramp, W. K., and Neuman, W. F., Amer. J. Physiol. **220,** 270 (1971).
14. Ramp, W. K., and Neuman, W. F., Fed. Proc. **31,** 708 (1972 Abstract).
15. Lowry, O. H., Roberts N. R., Wu, M., Hixon, W. S., and Crawford, E. J., J. Biol. Chem. **207,** 19 (1954).
16. Yoshimura, F., and Suzuki, T., Biochim. Biophys. Acta **410,** 167 (1975).
17. Lundholm, L., Mohme-Lundholm, E., and Vamos, E., Acta Physiol. Scand. **58,** 243 (1963).
18. Fishman, W. H., Am. J. Med. **56,** 617 (1974).
19. Arsenis, C., Hackett, M. H., and Huang, S. M., Calc. Tissue Res. **20,** 159 (1976).
20. Borgers, M., J. Histochem. Cytochem. **21,** 812 (1973).
21. Linde, A., and Magnusson, B. C., J. Histochem. Cytochem. **23,** 342 (1975).
22. Thomas, M. L., and Goz, B., Exp. Cell Res. **101,** 422 (1976).
23. Chase, L. R., Fedak, S. A., and Aurbach, G. D., Endocrinology **84,** 761 (1969).
24. Reynolds, J. J., and Dew, G. W., Experientia **33,** 154 (1977).

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