Effect of diphosphonates on adenosine 3':5'-cyclic monophosphate in mouse calvaria after stimulation by parathyroid hormone

in vitro

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(Received 28 November 1975)

Summary

1. The diphosphonates, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and disodium dichloromethylene diphosphonate (Cl₂MDP), inhibit bone resorption in animals and in explanted bone in tissue culture. The possibility that these effects might be due to inhibition of skeletal adenylate cyclase has been studied.

2. EHDP and Cl₂MDP, added for 30 min to the incubation medium at concentrations known to inhibit bone resorption, had no effect on basal content of adenosine 3':5'-cyclic monophosphate (cyclic AMP) of mouse calvaria incubated in vitro, nor did they inhibit the rise in cyclic AMP induced by bovine parathyroid hormone.

3. Pretreatment of mice for 3 days with Cl₂MDP also had no effect on cyclic AMP under basal conditions or after incubation of explanted calvaria with parathyroid hormone in vitro. EHDP under similar conditions slightly inhibited the increase induced by parathyroid hormone but had no effect on basal concentrations of cyclic AMP.

4. It is suggested that the inhibition of adenylate cyclase is not an essential feature of the reduction of bone resorption by diphosphonates, which may act by direct inhibitory effects on the dissolution of hydroxyapatite and perhaps by other unidentified effects on bone cells.

Key words: adenosine 3':5'-cyclic monophosphate, bone, dichloromethylene diphosphonate, diphosphonates, ethane-1-hydroxy-1,1-diphosphonate, parathyroid hormone.

Introduction

Parathyroid hormone is known to stimulate resorption of explants of bone in tissue culture (Gaillard, 1961; Raisz & Niemann, 1969; Raisz, Trummel & Simmons, 1972; Reynolds, Minkin, Morgan, Spycher & Fleisch, 1972) and in intact animals. One of the earlier effects of parathyroid hormone on bone in tissue culture is to produce a rise in bone adenosine 3':5'-cyclic monophosphate (cyclic AMP) content (Chase & Aurbach, 1970; Chase, Fedak & Aurbach, 1969; Aurbach & Chase, 1970; Herrmann-Erlee & Konijn, 1970; Peck, Carpenter, Messinger & DeBra, 1973; Rodan & Rodan, 1974; Smith & Johnston, 1974). Since both N⁶-2'-O-dibutyryl-3':5'-cyclic AMP (DAMP), as shown by Vaes (1968), Raisz, Brand, Klein & Au (1969), Heersche, Fedak & Aurbach (1971), Herrmann-Erlee & van der Meer (1974), and cyclic AMP can stimulate resorption in bone explants in culture, it has been suggested that parathyroid hormone may act on bone by causing changes in cyclic AMP content in bone cells. Diphosphonates, such as ethane-1-hydroxy-1,1-diphosphonate (EHDP), and dichloromethylene diphosphonate (Cl₂MDP), inhibit bone resorption both in tissue culture (Russell, Mühlbauer, Bisaz,
Williams & Fleisch, 1970; Reynolds et al., 1972; Reynolds, Murphy, Mühlbauer, Morgan & Fleisch, 1973; Minkin, Rabadjija & Goldhaber, 1974) and in experimental animals (Schenk, Merz, Mühlbauer, Russell & Fleisch, 1973; Gasser, Morgan, Fleisch & Richelle, 1972). EHDP has been used in man to reduce excessive bone turnover, notably in Paget's disease (Russell, Smith, Preston, Walton & Woods, 1974; Guncaga, Lauffenberger, Lentner, Dambacher, Haas, Fleisch & Olah, 1974; Altman, Johnston & Khairi, 1973).

Although the effects of diphosphonates on bone resorption have been thought to be due to inhibition of dissolution of apatite crystals, an effect which can be demonstrated readily on synthetic crystals in vitro (Fleisch, Russell & Francis, 1969), it is possible that other mechanisms may be involved. Diphosphonates closely resemble inorganic pyrophosphate (PPi), a product of the adenylate cyclase reaction, and it is possible therefore that they may interfere with production of cyclic AMP. Pilczyk, Sutcliffe & Martin (1972) have shown that both diphosphonates and PPi can reduce adenylate cyclase activity stimulated by fluoride or parathyroid hormone in a kidney-cell membrane preparation. Inhibition of fluoride- and glucagon-stimulated adenylate cyclase in isolated liver membranes has also been demonstrated (Eisman, Martin, Pilczyk, Legge & Sutcliffe, 1974).

In order to determine whether diphosphonates might act on bone by inhibiting adenylate cyclase we have examined the influence of diphosphonates given in vitro or in vivo on cyclic AMP content in mouse calvaria before and after stimulation by parathyroid hormone.

Materials and methods

Four-day-old mice from an NMRI strain bred in this Department were killed by decapitation. The calvaria were dissected out under a binocular microscope into Tyrode's solution containing 2-(N-2-hydroxyethyl)piperazin-N-yl)ethanesulphonic acid (HEPES buffer; 2.5 mmol/l) and bovine serum albumin (2.5 g/l), with a total osmolarity of 320 mosmol/l. Care was taken not to damage the periosteum during dissection. The calvaria, consisting of frontal and parietal bones, were divided in half and each half was dried on a paper towel and weighed on a Cahn Electrobalance. Immediately after, the halves were put back into the Tyrode–Hepes buffer–albumin solution and kept at room temperature until incubation.

After time-courses and dose-response curves to parathyroid hormone had been established, three groups of experiments were performed.

Experiment 1. One half of each calvaria was preincubated for 30 min at 37°C in Technicon Autoanalyzer sample cups containing 1 ml of the Tyrode–Hepes buffer–albumin solution (control half), and the other half in 1 ml of the same solution plus different amounts of either Cl2MDP or EHDP (treated half). After this 30 min preincubation, paired halves of these calvaria were incubated for a further 5 min either in a solution containing parathyroid hormone (5 units/ml) or in Tyrode–Hepes buffer–albumin without hormone. Both solutions contained no diphosphonates during the 5 min incubation.

Experiment 2. Calvaria were preincubated as above, but instead of the medium being changed after 30 min, parathyroid hormone was added for a further 5 min at a concentration of 5 units/ml, so that, in contrast to experiment 1, the diphosphonates were still present during the 5 min exposure to parathyroid hormone.

Experiment 3. Since it was possible that a preincubation time of 30 min was insufficient to allow the diphosphonates to be taken up by the bone, further experiments were done by injecting the diphosphonates subcutaneously into newborn mice. On each of 3 days, the animals received 161 µmol/kg daily of either Cl2MDP or EHDP dissolved in 50 µl of NaCl solution (165 mmol/l). The control animals received only the NaCl solution. On the fourth day, the calvaria were removed and incubated for 5 min without the 30 min preincubation. One-half of each calvaria was exposed to parathyroid hormone (5 units/ml) and the other to Tyrode–Hepes buffer–albumin solution alone. Under these conditions, in which diphosphonates are given to living newborn mice, it is known that bone resorption during subsequent incubation in vitro is markedly inhibited (Reynolds & Morgan, 1970).

After incubation, the calvaria were placed immediately into liquid nitrogen. The frozen pieces were homogenized with 1 ml of trichloroacetic acid (50 g/l) in a Dual glass homogenizer and the homogenates centrifuged at 12000 g for 5 min. The supernatant was extracted three times with twice its volume of ether saturated with water and then assayed for cyclic AMP by a protein-binding method (Gilmann, 1970). Phosphodiesterase added to the ether-extracted supernatant resulted in complete loss of measurable cyclic AMP. The binding curve, plotted
Diphosphonates and cyclic AMP in bone in vitro

as 1/c.p.m. against 1/concentration, gave a straight line. The binding protein for cyclic AMP and the inhibitor protein were both prepared from bovine skeletal muscle by the method of Gilman (1970).

Materials

Parathyroid hormone was partially purified native bovine parathyroid hormone (600 units/mg; Wilson Laboratories, Chicago, Ill., U.S.A.); Hapes buffer was from Serva (Heidelberg, Germany); bovine serum albumin (purified) was from Merck (Darmstadt, Germany); 8-[3H]-adenosine-3':5'-cyclic phosphate, ammonium salt (22.1 Ci/mmol), was from New England Nuclear Corp.; adenosine 3':5'-cyclic monophosphonic acid was from Sigma (St Louis, Mo., U.S.A.). The diphosphonates were kindly provided as their disodium salts by the Procter and Gamble Co. (Cincinnati, Ohio, U.S.A.). All other reagents used were of highest obtainable purity.

Results

Response to parathyroid hormone

A significant, approximately threefold increase in cyclic AMP content of the calvaria was obtained after adding parathyroid hormone to the medium. It was found that the maximum concentration of cyclic AMP was reached after 5 min incubation with the hormone so this incubation time of 5 min was chosen for further experiments.

Fig. 1 shows the dose–response curve for parathyroid hormone with an incubation time of 5 min. A significant but submaximal stimulation was always

![Fig. 1. Cyclic AMP content of mouse calvaria after 5 min incubation with various concentrations of bovine parathyroid hormone in vitro. Each point represents the mean value ± 2 SEM of five incubations.](image)

![Fig. 2. Effect of Cl₂MDP and EHDP on cyclic AMP content of calvaria incubated with and without parathyroid hormone. The stippled columns represent basal levels obtained after incubation in Tyrode–Hapes buffer–albumin solution alone, and the open columns represent the values obtained in the presence of parathyroid hormone (5 units/ml). In these experiments the diphosphonates were present with the calvaria for 30 min preincubation, but were not present during the subsequent 5 min incubation with or without parathyroid hormone. Each column shows the mean value ± 2 SEM of five incubations.](image)
obtained with 5 units/ml. This concentration, rather than 10 units/ml which produced a maximum response, was used in subsequent experiments, so that the conditions would be at their most sensitive for detecting stimulation or inhibition by diphosphonates.

Effect of diphosphonates added in vitro

Fig. 2 shows that 30 min preincubation with Cl₂MDP at concentrations of 8.1, 65 or 516 μmol/l had no effect on basal cyclic AMP content of the calvaria or on the rise induced by parathyroid hormone. Fig. 2 also shows that EHDP, added at the highest concentration of 516 μmol/l, had no effect on basal content of cyclic AMP or on the increase induced by parathyroid hormone.

In our hands these concentrations also inhibit bone resorption of calvaria in vitro. Thus addition of Cl₂MDP (65 μmol/l) to the tissue culture medium for 2 days inhibits resorption by 35% and addition of Cl₂MDP at 516 μmol/l by 48%. The addition of EHDP at 516 μmol/l similarly inhibits the resorption by 37%.

Fig. 3 shows that when diphosphonates were present both during the preincubation (30 min) and the final 5 min incubation period there was again no effect of either of the diphosphonates at a concent-

Effect of diphosphonates given to mice in vivo

Fig. 4 shows that neither Cl₂MDP nor EHDP given subcutaneously for 3 days to mice had an effect on cyclic AMP content of the calvaria when the bones were incubated in vitro for 5 min in control buffer after explantation. When parathyroid hormone was added to the buffer the usual rise in content of cyclic AMP occurred but was slightly but not significantly less than normal (Student’s t-test, P>0.05) in the calvaria from mice injected with EHDP.

Discussion

The basal amounts of cyclic AMP and the response to parathyroid hormone closely resemble those described by Chase et al. (1969). Neither of the diphosphonates, EHDP and Cl₂MDP, when added to calvaria in vitro or previously injected into mice, had any effect on basal content of cyclic AMP or on this response to parathyroid hormone. The only exception to this was the slight but not significant impairment of the response to the hormone in calvaria from mice treated with EHDP in vitro. The concentrations of diphosphonates used in vitro and in vivo were the
same as those previously found to inhibit bone resorption either when added in tissue culture or when injected into newborn mice. At the dose of EHDP at which some inhibition of parathyroid hormone-stimulated adenylate cyclase activity occurred, resorption is known to be blocked completely (Reynolds et al., 1972). However, Cl₂MDP, which inhibits resorption more strongly than EHDP, had no effect on content of cyclic AMP in any of the experiments. These experiments show that the inhibition by diphosphonates of resorption induced by parathyroid hormone is unlikely to be due to inhibition of skeletal adenylate cyclase.

These results are in contrast with those of DeLong, Feinblatt & Rasmussen (1971), who showed that high doses of PP₃ infused into rats inhibited bone resorption and at the same time inhibited renal production of cyclic AMP. Furthermore, Pilczyk et al. (1972) and Eisman et al. (1974) have demonstrated inhibition by diphosphonates of renal and liver adenylate cyclase responses to parathyroid hormone and glucagon respectively. The lowest concentrations required to produce some inhibition were 2.5 μmol/l, although maximal inhibition required doses in the range 0.1–1.0 mmol/l. This corresponds to the highest concentrations of 0.516 mmol/l used here, which are capable of inhibiting bone resorption almost completely but have no effect on content of cyclic AMP. The experiments of Eisman et al. (1974) were done on isolated plasma membranes rather than on an intact tissue as in the present experiments. Broken-cell preparations may be more sensitive to inhibition by diphosphonates; it is possible that inhibition in intact cells would require uptake of diphosphonates by cells, and it is not known whether this occurs. The concentration of diphosphonates around bone cells is probably not the same as those added to the medium because of adsorption by hydroxyapatite crystals. It is possible also that there may be differences in the sensitivity of the cyclases from different tissues. However, even with kidney adenylate cyclase, it appears that in humans the increase in urinary cyclic AMP and in phosphate clearance induced by parathyroid hormone is unaltered by prior treatment with EHDP (Recker, Hassing, Lau & Saville, 1973).

It could be argued that the response to parathyroid hormone might have been impaired if the diphosphonates had been given for longer or if the effect of the hormone had been measured at another time, particularly since the cyclic AMP response is transient and occurs a long time before increased bone resorption takes place. Furthermore the calvaria contain several different types of cell, and it is possible that their response may vary in such a way that, although no overall change in cyclic AMP takes place, there may be changes within particular cell populations. Indeed, studies on isolated bone cells show that parathyroid hormone increases cyclic AMP content in cell populations unlikely to contain many osteoclasts (Peck et al., 1973) and that the effect varies according to the site from which the cells are derived (Smith & Johnston, 1974; Wong & Cohn, 1975). The role of cyclic AMP in parathyroid hormone-mediated resorption is still unclear, especially since other agents (e.g. 1,25-dihydroxycholecalciferol, unpublished results, and vitamin A and 25-hydroxycholecalciferol) which promote bone resorption can do so without causing a detectable increase in cyclic AMP (Mahgoub & Sheppard, 1975). Moreover, calcitonin, which inhibits bone resorption, potentiates rather than inhibits the action of parathyroid hormone on adenylate cyclase, perhaps because it acts on a separate cell population (Wong & Cohn, 1975). It is possible that the changes in cyclic AMP in response to hormone are related to the control of cell division (Burger, Bombik, Breckenridge & Sheppard, 1972; Otten, Johnson & Pastan, 1971) rather than to the stimulation of the resorption process itself. Therefore these results show that the inhibition of parathyroid hormone-induced bone resorption by EHDP and Cl₂MDP does not induce an early inhibition of the adenylate cyclase reaction. It is nevertheless possible that the diphosphonates inhibit bone resorption by an independent action on cells. There are changes in osteoclast morphology and matrix biosynthesis (Minkin et al., 1974; Schenk et al., 1973; Doty, Jones & Finerman, 1972) under the diphosphonates but it is impossible to know whether these are direct effects or changes brought about indirectly, for example, by changing the local ionic environment as a result of inhibition of apatite dissolution.

Acknowledgments

This work has been supported by the Swiss National Foundation for Scientific Research (KF 6 and 3.121.73), by the US Public Health Service (AM-07266), and by the Procter and Gamble Co., U.S.A.

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