JCI The Journal of Clinical Investigation

Differential Action of the Bisphosphonates (3-Amino-1-Hydroxypropylidene)-1,1-Bisphosphonate (APD) and Disodium Dichloromethylidene Bisphosphonate (Cl_2MDP) on Rat Macrophage-mediated Bone Resorption In Vitro

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J Clin Invest. 1982;70(5):927-933. https://doi.org/10.1172/JCI110704.

Research Article

The bisphosphonates (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD) and disodium dichloromethylidene bisphosphonate (Cl_2MDP) effectively inhibit the accelerated bone resorption associated with some skeletal disorders, e.g., Paget's disease. However, it has not been established whether these compounds exert their inhibitory effect by rendering the bone mineral more resistant to degradation, by diminishing the activity of resorbing cells, or through some combination of both activities. In this study, we have tested these possibilities using an in vitro resorption assay system consisting of elicited rat peritoneal macrophages co-cultured with particles of ^{45}Ca-labeled, devitalized rat bone. This assay system permits the quantitative assessment of the action of APD and Cl_2MDP on the two major phases of bone resorption (cell-substrate attachment and osteolysis) under circumstances where the drugs are present continuously or, most importantly for the issues in question, after the separate pretreatment of the particles or the resorbing cells.

Our data indicate that (*a*) Both APD and Cl_2MDP at concentrations ≥5 × 10^{-6} M diminish macrophage-mediated ^{45}Ca release (i.e., bone resorption) in a log dose-dependent fashion. (*b*) A 10-min pretreatment of bone particles with either bisphosphonate (P-C-P) similarly inhibits resorptive activity, but is most pronounced with Cl_2MDP. However, only APD is effective in reducing resorption when cells are preincubated (for 24 h) with P-C-P. (*c*) In cultures containing both labeled and unlabeled bone, significant inhibition occurs only when […]

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ABSTRACT The bisphosphonates (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD) and disodium dichloromethylidene bisphosphonate (Cl_2MDP) effectively inhibit the accelerated bone resorption associated with some skeletal disorders, e.g., Paget's disease. However, it has not been established whether these compounds exert their inhibitory effect by rendering the bone mineral more resistant to degradation, by diminishing the activity of resorbing cells, or through some combination of both activities. In this study, we have tested these possibilities using an in vitro resorption assay system consisting of elicited rat peritoneal macrophages co-cultured with particles of ^{45}Ca-labeled, devitalized rat bone. This assay system permits the quantitative assessment of the action of APD and Cl_2MDP on the two major phases of bone resorption (cell-substrate attachment and osteolysis) under circumstances where the drugs are present continuously or, most importantly for the issues in question, after the separate pretreatment of the particles or the resorbing cells.

Our data indicate that (*a*) Both APD and Cl_2MDP at concentrations $\geq 5 \times 10^{-6}$ M diminish macrophage-mediated ^{45}Ca release (i.e., bone resorption) in a log dose-dependent fashion. (*b*) A 10-min pretreatment of bone particles with either bisphosphonate (P-C-P) similarly inhibits resorptive activity, but is most pronounced with Cl_2MDP. However, only APD is effective in reducing resorption when cells are preincubated (for 24 h) with P-C-P. (*c*) In cultures containing both labeled and unlabeled bone, significant inhibition occurs only when the labeled particles are coated with P-C-P (indicating that the action of P-C-P-treated bone is highly localized). (*d*) P-C-P does not diminish cell-bone particle attachment, an essential step in the resorptive process. On the other hand, delaying the addition of P-C-P until after cell-bone attachment is completed significantly reduces the resorption-inhibiting effect of these compounds. (*e*) Cl_2MDP reduces culture DNA content in proportion to its inhibitory effect on resorption, and both the inhibitory and cytotoxic actions of this P-C-P are dependent upon the presence of bone. On the other hand, APD is cytotoxic only at very high concentrations (10^{-4} M), acts independently of the presence of bone, and inhibits resorption without killing cells.

We conclude that the mechanisms of action of APD and Cl_2MDP are markedly different. Cl_2MDP is a potent cytotoxin in the presence of bone and apparently exerts its inhibitory effect in this manner. APD is noncytotoxic at levels adequate to suppress resorption and, therefore, must inhibit macrophage activity by some other mechanism. Neither P-C-P appears to limit resorption by decreasing the solubility of mineralized bone matrix.

Received for publication 22 February 1982 and in revised form 9 July 1982.

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INTRODUCTION

Bisphosphonates (P-C-P)[1] are nonbiodegradable analogues of pyrophosphate (PP_i) with a high binding affinity for hydroxylapatite crystals (1). Administration of P-C-P to experimental animals rapidly curtails bone resorption (2–4), and it is for this reason that these compounds are used in the treatment of clinical disorders characterized by increased bone degradation. For example, both disodium (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD) and disodium dichloromethylidene bisphosphonate (Cl_2MDP) are effective in decreasing bone resorption in Paget's disease and in the osteolytic lesions associated with neoplastic bone disease (5–8). Interestingly, APD effectively decreases bone resorption in vivo at much lower doses than does Cl_2MDP. There is no clear explanation for this difference in potency, but it may relate to the observed effects of APD on the mononuclear phagocyte system (9). Cells of the mononuclear phagocyte system (MPS) have the potential to regulate osteoclast activity (e.g., by producing prostaglandin E_2) and APD might alter this aspect of MPS function.

The basis for the resorption-inhibiting effects of P-C-P remains unknown, but given the binding affinity of these compounds for hydroxylapatite crystals, they may well act subsequent to accumulation on bone mineral surfaces. Once present on such surfaces, they may exert their inhibitory effect by (*a*) directly decreasing mineral solubility and ultimately the resorbability of bone matrix, (*b*) inhibiting the attachment of osteoclasts and other potential resorbing cells (macrophages, tumor cells) to bone surfaces, or (*c*) diminishing the resorption-related and/or general metabolic activity of osteoclasts and other osteolytic cells. Alternatively, circulating P-C-P may act directly on cells to block cell-bone attachment or resorption, without prior binding to mineral surface.

At present, there is little information regarding the relative biological significance of each of these possible mechanisms of P-C-P inhibition. It is known, for example, that P-C-P will inhibit the growth and dissolution of hydroxylapatite crystals in vitro (10, 11). In addition, P-C-P have been shown to modify the metabolic activity of several different cell types grown in culture (12, 13). However, in none of these cases have bone matrix (or mineral) and a cell with osteolytic potential been separately treated with P-C-P and then combined in such a way as to assess the subsequent action of these compounds on the resorptive process.

In this study, we have explored the mechanism of action of Cl_2MDP and APD using a recently developed, macrophage-based assay system for bone resorption (14). With this system, it is possible to independently treat resorbing cells (elicited peritoneal macrophages) and bone mineral (devitalized rat bone particles) with P-C-P and to quantify the results of such treatment on the ability of the macrophages to bind and resorb bone. Our data indicate that APD and Cl_2MDP do not alter the attachment of cells to bone but do inhibit macrophage-mediated resorption in a dose-dependent fashion. In the case of Cl_2MDP, this inhibition is bone mineral dependent, occurs primarily subsequent to the accumulation of P-C-P on the bone crystal surface, and appears to be a manifestation of the cytotoxicity of this P-C-P. In the case of APD, on the other hand, the P-C-P is cytotoxic only at high concentration, acts independently of the presence of bone and inhibits resorption, at least in part, by a mechanism not yet defined but probably dependent upon the direct action of APD on cells. Neither P-C-P appears to affect resorption by reducing the solubility of the mineral phase of bone.

METHODS

Binding and resorption assays are performed as described (14) and are summarized as follows: Elicited peritoneal adherent cells (MØ) were obtained from rats (Sprague-Dawley, ±150 g) 3 d after a peritoneal injection with 10% Brewer's thioglycollate medium. The exudate cells were allowed to attach for 1 h to the wells of Costar dishes (5×10^5 cells/16-mm well) in 0.5 ml modified Eagle's minimal essential medium (α-MEM) buffered to pH 7.4 with 3*N*-morpholinopropanesulfonic acid. The cultures were rinsed, and α-10 medium (bicarbonate-buffered α-MEM supplemented with 10% fetal calf serum) was added to each well. The plates were incubated at 37°C in humidified air and 5% CO_2 for an additional 18–23 h, and then vigorously rinsed to remove nonadherent cells. The remaining adherent cells were 95–98% macrophages as assessed by phagocytosis and nonspecific esterase staining.

Particles (≤25 μm) of devitalized rat bone, labeled in vivo with ^{45}Ca, were suspended in α-10 medium and were added to the macrophage cultures to yield a final concentration of 0.115 mg/well. Binding of the bone particles to the cells was assessed after 2 h of incubation by recovering the nonadherent particles from the culture surface and determining the isotopic content of the adherent and nonadherent particle fractions. Bone resorption (expressed as net percentage of ^{45}Ca release) was routinely determined after 96 h incubation from the percentage of ^{45}Ca released into the medium as a consequence of macrophage activity, and was corrected for physicochemical exchange of ^{45}Ca that occurs when bone is incubated in the absence of cells. The results of both binding and resorption assays were derived from six replicate cultures for each variable and are presented as mean±SEM. Experiments were generally repeated two to three times before the findings were accepted as valid.

The bisphosphonates APD and Cl_2MDP, and PP_i were

[1] *Abbreviations used in this paper*: APD, (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate; Cl_2MDP, disodium dichloromethylidene bisphosphonate; α-MEM, Eagle's minimal essential medium; MØ, adherent cell; MPS, mononuclear phagocyte system; P-C-P, bisphosphonate(s); PP_i, inorganic pyrophosphate.

dissolved in 0.9% saline and diluted in α-10 medium to the desired concentrations before addition to the cultures. Alternatively, bone particles are pretreated by incubating them for 10 min in solutions of P-C-P, PP_i, or phosphate buffer, resuspended in α-10 medium and added to the cultures. Pretreatment of the MØ was achieved by incubating the cells for 24 h in P-C-P contained in α-10 medium, and then rinsing and adding labeled bone to the cultures. Cell number and viability were indirectly assessed by measuring the DNA content of the MØ population using the ethidium bromide technique described by Karsten and Wollenberger (15).

RESULTS

APD and Cl_2MDP, when present throughout the entire assay period, reduced net ^{45}Ca release from the bone particles in a log dose-dependent fashion. In the representative experiment shown in Fig. 1, significant inhibition of resorption was achieved by both compounds at 5×10^{-6} M ($P < 0.01$). The molar potency of the two P-C-P was equal. On the other hand, the passive efflux of ^{45}Ca from bone particles incubated in the absence of cells, which accounted for ~20% of the radioactivity in the culture medium, was not altered by either APD or Cl_2MDP (data not shown).

Inhibition of ^{45}Ca release also occurred when particles were preincubated for 10 min in solutions containing APD or Cl_2MDP before being introduced into cultures. Using this approach, Cl_2MDP was more effective, on a molar basis, than APD, whereas pretreatment in control solutions of PP_i or phosphate buffer was without effect (Fig. 2). When compared with its continuous presence in cultures, the dose-response curve for preincubation of bone particles with Cl_2MDP was shifted to the right by about half an order of magnitude (Fig. 3). In marked contrast, only APD was effective in reducing macrophage-mediated resorption under circumstances where the cells were preincubated with P-C-P for 24 h (Table I).

Additional evidence for a direct involvement of bone surfaces in mediating the inhibitory action of P-C-P was obtained from experiments in which ^{45}Ca-labeled and unlabeled bone were introduced concurrently into replicate MØ-containing cultures. In some

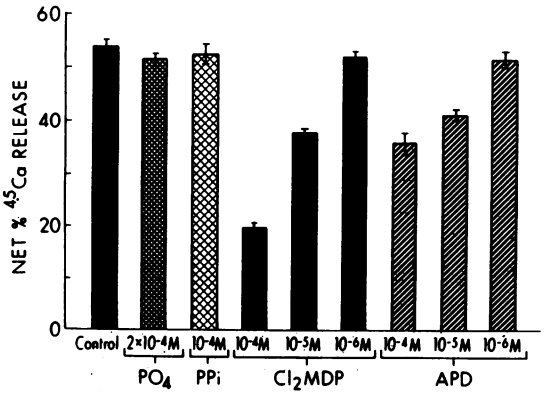

FIGURE 2 Effect of 10-min preincubation of devitalized ^{45}Ca-labeled bone particles with APD, Cl_2MDP, PP_i or PO_4, on ^{45}Ca release by MØ. All values represent mean±SEM from six determinations.

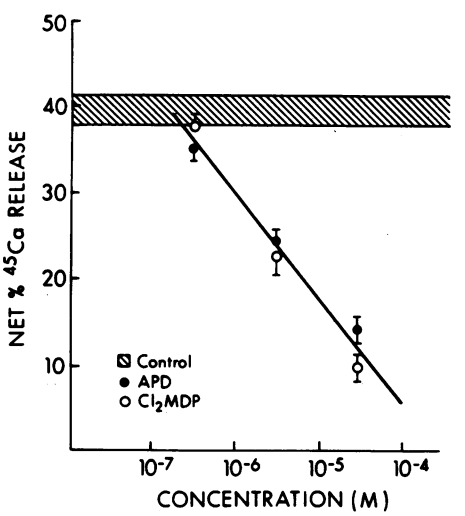

FIGURE 1 P-C-P inhibition of ^{45}Ca release by MØ from devitalized ^{45}Ca-labeled bone particles after 96 h incubation. APD and Cl_2MDP were continuously present at the concentrations indicated. Net percent ^{45}Ca release was calculated by subtracting the ^{45}Ca release from cultures with bone particles only (passive efflux) from the ^{45}Ca release in the cultures containing MØ and bone particles. All values represent mean±SEM from six determinations.

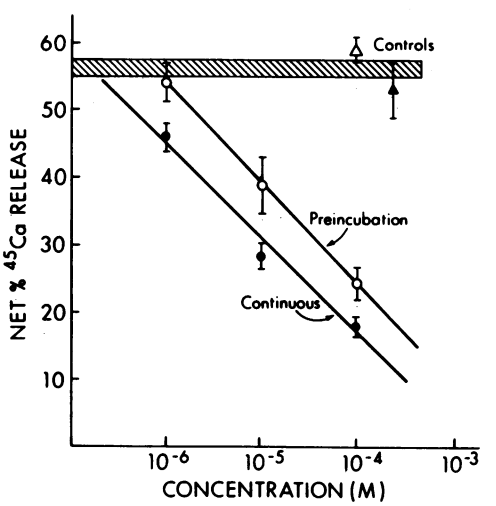

FIGURE 3 Comparison of bone particle pretreatment with continuous exposure to Cl_2MDP on macrophage-mediated resorption. Additional controls were bone particles preincubated with PO_4-buffer (2×10^{-4} M, open triangles) and PP_i (10^{-4} M, closed triangles). Note the relatively small difference in dose-response between the two modes of introducing P-C-P. All values represent the mean±SEM for six replicate cultures.

TABLE I

Effect of Cl_2MDP and APD on Mϕ-mediated Bone Resorption when Mϕ Were Preincubated with P-C-P for 24 h before the Introduction of Bone

Control	Net ^{45}Ca release
	%
	39.5±1.9
5×10^{-5} M APD	17.3±3.9*
5×10^{-6} M APD	39.5±1.7
5×10^{-7} M APD	37.9±1.0
5×10^{-5} M Cl_2MDP	43.7±2.4
5×10^{-6} M Cl_2MDP	41.1±1.0
5×10^{-7} M Cl_2MDP	40.6±1.6

All values are means±SEM from six determinations.
* Significantly different from control, $P < 0.01$.

instances, the labeled particles were pretreated with P-C-P before being added to the wells; in other cases, it was the unlabeled bone that was coated with P-C-P. If P-C-P act primarily by their presence at the cell-bone interface, then only those wells containing labeled bone and P-C-P should show evidence of reduced resorptive activity. In the experiment depicted in Fig. 4, the decrements in apparent resorptive activity were only significant in cultures containing labeled bone pretreated with P-C-P.

We have previously demonstrated that binding of bone by MØ is a prerequisite for resorption and that a lag period of ~8 h exists between the initiation of

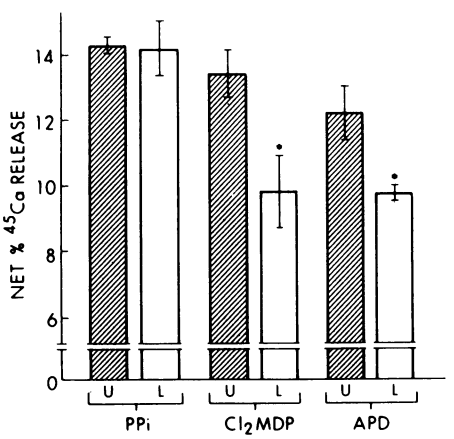

FIGURE 4 The co-culture of labeled and unlabeled bone "coated" with Cl_2MDP or APD. In some instances the labeled bone (L) was pretreated (10 min) with 10^{-5} M Cl_2MDP or APD; in other cases the unlabeled bone (U) was pretreated. Notice that significant (*) inhibition of resorption (^{45}Ca release) was evident only in those instances where the labeled particles were coated with P-C-P ($P < 0.01$, Cl_2MDP; $P < 0.001$, APD). All values represent the mean±SEM from six determinations.

TABLE II

Effect of Cl_2MDP and APD on the 2-h Attachment of Bone Particles to Cells

Control	Particles bound
	%
	80.5±0.9
5×10^{-5} M APD	81.0±0.7
5×10^{-7} M APD	81.8±0.9
5×10^{-5} M Cl_2MDP	82.5±0.4
5×10^{-7} M Cl_2MDP	83.2±0.7

All values are means±SEM from six determinations.

binding and the onset of osteolysis (14, and unpublished observations). Table II shows that neither compound interferes with the binding of bone by the cells. However, deferring the introduction of P-C-P until after maximum cell-particle binding is achieved and bone degradation begins, significantly reduces the resorption-inhibiting effect of these compounds (Fig. 5). This effect was most notable with Cl_2MDP where an 8-h delay totally eliminated the suppressive effect of this P-C-P. APD, on the other hand, continued to be

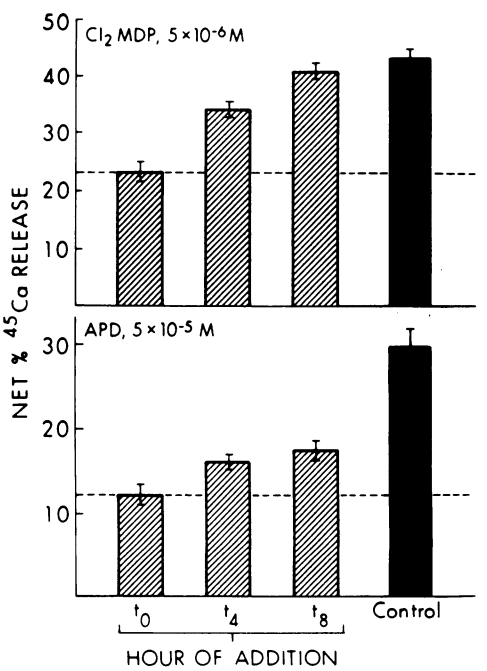

FIGURE 5 Effect of delayed addition of APD and Cl_2MDP on the inhibition of ^{45}Ca release by MØ from devitalized bone particles after 96 h incubation. Note that in all cases, delaying the introduction of P-C-P renders the compounds significantly less inhibitory ($P < 0.01$ to $P < 0.001$), but that the effect is most pronounced with Cl_2MDP. All values represent the mean±SEM from six determinations.

strongly inhibitory even when introduced after the particle binding phase was completed.

Chambers (18) recently reported that high concentrations of Cl_2MDP are cytotoxic for murine macrophages. This cytotoxicity was also evident in rat peritoneal cells and likely accounts for all the inhibition of MØ-mediated resorption exhibited by this P-C-P in vitro. For example, as can be seen in Fig. 6, in cultures containing bone particles and treated with Cl_2MDP, for the 96-h assay period, there is a near proportionality between the degree of cytotoxicity (loss of DNA) and the extent of the inhibition. We noted earlier the effectiveness of pretreating bone particles with Cl_2MDP in suppressing resorption (Fig. 2). The critical importance of the bone particles in contributing to the action of Cl_2MDP was further documented by experiments in which cytotoxicity was shown to vary as a function of both the amount and particle size of bone added to the cultures. From the experiment depicted in Fig. 7, it can be seen that the decrement in the amount of DNA increases with increasing amounts of bone, and that smaller bone particles are approximately an order of magnitude more effective in enhancing cytotoxicity than are larger ones.

The relationship between cytotoxicity and the inhibition of resorption was much less certain with regard to APD and Cl_2MDP. For example, in contrast to Cl_2MDP, cultures treated continuously with APD exhibit relatively greater inhibition of resorption than loss of cells (Fig. 6). Moreover, and again in distinction

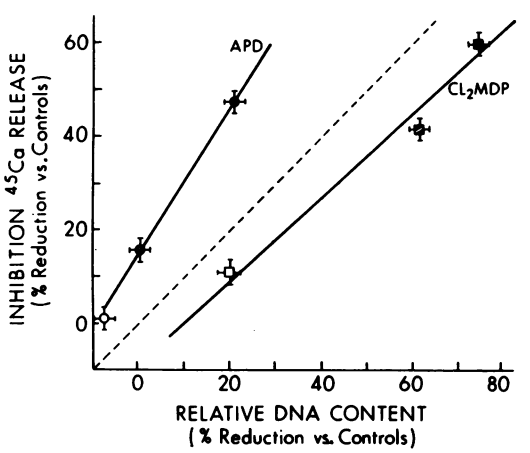

FIGURE 6 Relationship between the inhibition of ^{45}Ca release and the reduction in DNA content of the MØ population in incubations containing bone particles. The concentrations of APD (circles) and Cl_2MDP (squares) were 10^{-6} M (open symbols), 10^{-5} M (striped symbols), and 10^{-4} M (filled symbols). Notice that with APD, the inhibition of resorption is greater than would be expected on the basis of cytotoxicity (loss of DNA) alone. All values represent the mean±SEM from six determinations.

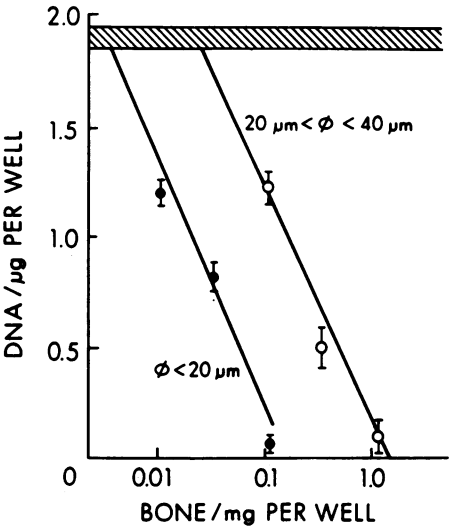

FIGURE 7 Cl_2MDP cytotoxicity as a function of the amount and particle size of bone added to macrophage cultures. (In this figure ϕ denotes diameter of bone particles.) Note that increasing the amount of bone leads to increased cytotoxicity (measured as the loss of DNA per well) and that smaller bone particles are more effective in enhancing cytotoxic activity than are larger ones. Each value represents the mean±SEM from six replicate cultures.

to Cl_2MDP, the degree of cell death in APD-treated cultures was independent of the mode of presentation of the P-C-P and whether or not bone was present in the wells (Table III). Finally, and perhaps most importantly, APD showed significant inhibition of resorption at concentrations lower than those needed to

TABLE III
Effect of APD and Cl_2MDP on DNA Content of Cultures after 96-h Incubation

	DNA/well, μg		
Control	2.76±0.07		
	Continuous exposure, with bone particles	Preincubation, with bone particles	Preincubation cells (no bone particles)
DAP			
10^{-6} M	3.02±0.09	2.96±0.04	2.85±0.10
10^{-5} M	2.80±0.05	2.74±0.06	2.64±0.07
10^{-4} M	2.18±0.07‡	2.21±0.02‡	1.96±0.10‡
Cl_2MDP			
10^{-6} M	2.20±0.04‡	2.53±0.06***	—
10^{-5} M	1.03±0.10‡	1.63±0.06*‡*	—
10^{-4} M	0.71±0.10‡	0.78±0.07*‡*	—

All values are means±SEM from six determinations.
* Significantly different from control $P < 0.05$.
‡ Significantly different from control, $P < 0.001$.

affect cell viability (loss of DNA; c.f., Fig. 6), and with a different time course of activity (Fig. 8).

DISCUSSION

We mentioned previously that P-C-P bind to and limit the in vitro solubility of hydroxylapatite (bone) crystals (1, 10, 11). It follows from this that P-C-P may inhibit bone resorption by directly increasing the resistance of the mineral phase of bone to degradation (16). However, the inhibition of resorption by P-C-P is also associated with dramatic changes in both osteoclast morphology and in the size of the osteoclast population (2–4, 17), indicating that these compounds exert a profound effect upon resorbing cells as well as upon the bone mineral.

To gain further insight into the relative importance of P-C-P action on mineralized bone matrix and resorbing cells, we have assessed the effect of these compounds using an in vitro assay system that permits the independent treatment of the cellular and matrical components of bone resorption. Specifically, we have determined the binding and resorption of isotopically labeled rat bone particles by elicited rat macrophages under conditions where the drugs were present continuously and following the preincubation of the cells or bone particles with P-C-P.

The suppression of ^{45}Ca release in the continuous presence of Cl_2MDP or APD documents the inhibitory effect of P-C-P on bone resorption in the macrophage-resorption system. Moreover, the lack of change in the passive movement of ^{45}Ca in the presence of either compound argues that this inhibition cannot be explained simply by crystal stabilization. Similarly, the effect of P-C-P on resorption does not appear to be due to a diminution in the avidity with which resorbing cells bind the bone particles, a step that we have previously shown to be essential in the resorptive process (14).

On the other hand, our data do indicate that the binding of P-C-P to bone mineral very likely plays an important role in the skeletal action of these compounds, at least with regard to Cl_2MDP. For example, pretreatment of ^{45}Ca-labeled bone particles with Cl_2MDP suppresses subsequent resorptive activity almost as effectively as having the P-C-P present continuously in the culture medium. In addition, the observation that the inhibitory action of P-C-P is reduced by delaying their introduction into the culture suggests that the compounds lose at least some of their effectiveness if they are prevented from accumulating on crystals' surfaces until cell-matrix attachment is completed and resorption initiated. This observation is particularly striking in the case of Cl_2MDP, where an 8-h delay entirely mitigates its inhibitory action. Finally, the data from the experiment utilizing treated unlabeled and labeled bone further establishes that mineral-bound P-C-P can directly suppress resorption without prior release of P-C-P from the bone surface or of some secondary effector substance (monokine) from macrophages.

The proportional diminution in the DNA content of adherent macrophage populations and resorptive activity indicates that cytotoxicity is largely responsible for the inhibitory effect of Cl_2MDP on bone resorption, as has been previously suggested (18). This cytotoxicity is dependent upon the presence of bone and, as we have shown here, increases when additional

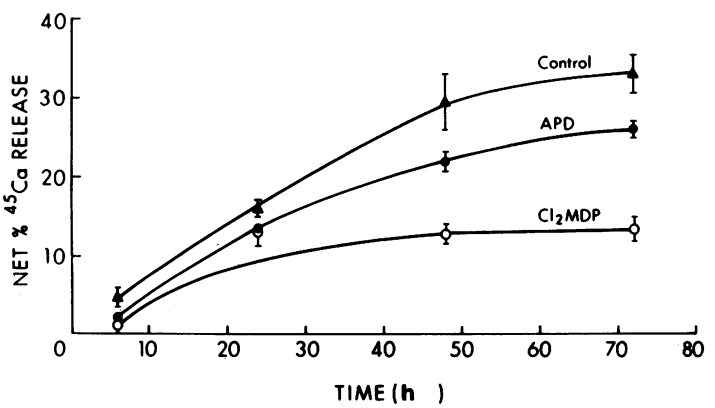

FIGURE 8 Time course of the inhibition of resorption by 5×10^{-5} M Cl_2MDP and APD. Note that both P-C-P show marginal inhibitory activity within the first 24 h of culture and significant inhibition by 48 h. Cl_2MDP is clearly more potent than APD and essentially curtails all resorptive activity after the 1st d of incubation. In this experiment only Cl_2MDP proved cytotoxic, reducing culture DNA values by 72 h to 1.38 μg/well vs. 2.13±0.13 for controls and 2.05±0.15 for APD-treated cultures. Each value represents the mean±SEM from six replicate cultures.

bone particles are added to the cultures. We noted above the importance of bone surfaces in "delivering" P-C-P. This aspect of P-C-P action is reinforced by the observation that smaller bone particles, with their substantial surface-to-volume ratio, are significantly more effective in enhancing cytotoxicity than are larger particles (even if the latter are present in much greater amounts).

In contrast to Cl_2MDP, APD does not appear to inhibit macrophage-mediated resorption solely by killing the resorbing cells. For example, there is a poor correspondence between the loss of adherent macrophages and the inhibition of resorption in cultures treated with APD, and, in fact, significant inhibition is usually observed in the absence of a demonstrable cytotoxicity. Moreover, when cytotoxicity is encountered, it is at very high concentrations of APD (10^{-4} M) and is independent of both the mode of introduction of the P-C-P and whether or not bone is present in the cultures.

APD and Cl_2MDP do not behave identically in vivo (9) and other differences in the mechanisms of action of these compounds are evident in the present in vitro study. Cl_2MDP appears to work as a cytotoxin in the presence of bone, and this seems adequate to account for the diminution in macrophage-mediated resorption reported above and, perhaps, in animals treated with this P-C-P. APD, on the other hand, suppresses resorption without killing the resorbing cells and without the intervention by bone. This latter effect is, perhaps, most notable in the delayed introduction experiments, where APD continues to exhibit significant inhibitory action even after the completion of cell-bone binding. Thus, while the precise mechanism of action of APD remains unknown, the fact that it can act directly on sensitive cells may account for the relatively greater in vivo potency exhibited by this compound when compared to other P-C-P, e.g., Cl_2MDP and EHDP (1-hydroxyethylidene-1,1-bisphosphonate).

ACKNOWLEDGMENTS

The authors wish to acknowledge the expert technical assistance of Jenny Sadow and Lianne van der Wee-Pals. APD and Cl_2MDP were kindly donated by Henkel KGaA, Düsseldorf, West Germany.

This work was supported, in part, by National Institutes of Health grant DE-05413 and DE-04629 and by the Foundation for Medical Research (FUNGO), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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