Effect of different inhibitors on nonspecific alkaline phosphatase in the microsomal fraction from isolated odontoblasts

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In the microsomal fraction isolated from rat incisor odontoblasts, the influence of several inhibitors on *p*-nitrophenyl phosphatase activity (*p*-NPPase) was demonstrated at pH 10.3 and compared with the influence at pH 8.8. The inhibiting effect of EDTA on the *p*-NPPase activity was reversed by equimolar concentrations of Zn^{2+}. The inhibitory effect of three diphosphonates, ethane-1-hydroxydiphosphonate (EHDP), methanediphosphonate (MDP), and dichloromethanediphosphonate (Cl_2MDP) was equal to that of a chelating agent. Tetramisole and levamisole exerted an inhibitory effect that was different from the complexing type. L-Cystein, but not L-ascorbic acid, inhibited *p*-NPPase activity with a maximum inhibitory effect at pH 10.5. □ *Enzyme biochemistry; EDTA; diphosphonates; tetramisoles;* L-*ascorbic acid;* L-*cysteine*

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Nonspecific alkaline phosphatase (APase, EC 3.1.3.1) has for many years been associated with hard tissue formation because of its high activity in areas of mineralization. Even though the true substrate for APase is not known, the enzyme is believed to take part in the mineralization process in part by dephosphorylating inorganic pyrophosphate (PP_i) (8, 13, 18, 22).

APase has earlier been reported to be Mg^{2+}-dependent with Zn^{2+} or Co^{2+} as cofactor (1, 11, 17, 19, 20). In the absence of Mg^{2+}, Ca^{2+} has been shown to activate the enzyme (6).

Since the APase activity using PP_i as substrate is Mg^{2+}-dependent (11, 20), the interpretation of the inhibition of complexing substances is very difficult.

The aim of this study was therefore to study the effect of various complexing substances such as EDTA and diphosphonates and of some inhibitors such as the tetramisoles L-cysteine and L-ascorbic acid on APase from rat incisor odontoblasts, using *p*-nitrophenyl phosphate (*p*-NPP) as substrate, since this reaction is not Mg^{2+}-dependent (12).

Materials and methods

Male Sprague-Dawley rats with a body weight of 200 g were used. Odontoblasts from the upper and lower incisors were dissected free as described earlier (15). The microsomal fraction from the isolated odontoblasts was prepared according to the method of Granström et al. (14).

The reactions were performed in 3-ml glass test tubes. The reaction mixture had a total volume of 60 µl. Of this, 40 µl consisted of 0.1 M glycine-NaOH buffer, pH 8.8 or 10.3, which was 4.5 mM with respect to disodium *p*-nitrophenyl phosphate (Merck AG, Darmstadt, FRG) and $MgCl_2$, unless otherwise stated. This concentration of *p*-NPP and $MgCl_2$ was used to give a final concentration of 3 mM when all reagents were added.

The rest of the reaction volume (10 µl) was used for the addition of inhibitory substances, which were prepared as follows. The inhibitory substances were dissolved in 10 ml 0.1 M glycine-NaOH buffer of appropriate pH, and adequate dilution series were performed. The final pHs of the solutions were

checked, and when EDTA or the disphosphonates (see below) were used, it was necessary to start the dilution series with a higher pH of the buffer to compensate for the low pH obtained at higher concentrations of the inhibitory substance.

When Zn^{2+} (chloride salt, Merck AG) was present, this was added in appropriate amounts to the original 40-µl portion of the reaction mixture; Mg was then omitted, and EDTA was added as described above. When the effects of L-cysteine and L-ascorbic acid were studied, appropriate amounts of the respective substances were added to 10 ml buffer of the respective pH, after which the pH was rechecked.

The enzyme reaction was started by the addition of 10 µl of the microsomal fraction (0.2 mg protein, w/v) dissolved in 0.1 M glycine-NaOH buffer of the respective pH. After incubation at 37°C for 30 min, the reaction was stopped by cooling on ice, followed by the addition of 0.5 ml 0.02 M NaOH. The reaction mixture was transferred to quartz microcells (type Mt 2; Carl Zeiss), and the liberated *p*-nitrophenol was measured at 420 nm, using a standard spectrophotometer (PMQ II, Carl Zeiss), equipped with a housing for microcells.

References consisted of samples with the same mixtures, in which the microsomal fraction was exchanged for 10 µl 0.1 M glycine-NaOH buffer. The optical turbidity of 10 µl of microsomal fraction was subtracted from all measurements.

The protein concentration of the microsomal fraction was measured by the method of Lowry et al. (16), using albumin as a standard, and on the basis of this the enzyme activities were given as µmol liberated phosphate (P_i) $\times$ mg protein^{-1} $\times$ min^{-1}.

In the experiments the following inhibitory substances were used: ethylenediaminetetraacetic acid (EDTA; Merck AG); methanediphosphonate (MDP), ethane-1-hydroxy-1,1-diphosphonate (EHD), and dichloromethanediphosphonate (Cl_2MDP), all kindly supplied by Henkel and Co, GmbH, Düsseldorf, FRG); levamisole, dexamisole, L-*p*-bromotetramisole, and D-*p*-bromotetramisole (Janssens Pharmaceutica, Belgium); and L-ascorbic acid and L-cystein (Sigma Chemical Co., St. Louis, Mo., USA).

Results

In Fig. 1 the influence of various concentrations of EDTA at the optimal pH of 10.3 on the *p*-NPPase activity is shown. Adding EDTA to a concentration of 2.5 mM inhibited the enzyme activity by 80%. Higher concentrations of EDTA resulted in a maximal inhibition of 85% of the enzyme activity. This inhibition was much less pronounced at pH 8.8; then the maximal inhibition of enzyme activity was 35%. Omitting Mg^{2+} reduced the *p*-NPPase activity at pH 10.3 by 10% at EDTA concentrations below 2.5 mM following the same slope but did not affect the enzyme activity noticeably at pH 8.8 (data not shown).

Utilizing the known inhibiting effect of EDTA on the *p*-NPPase activity, it was found that addition of Zn^{2+} to the medium in different amounts restored the inhibited *p*-NPPase activity. Added Zn^{2+} had a pronounced effect on the *p*-NPPase activity at pH 10.3, whereas the effect at pH 8.8 was much lower (Figs. 2 and 3.) However, at both pHs the original activity could be almost completely restored by the addition of Zn^{2+} in amounts that were equimolar to EDTA in the medium. It can also be seen (Figs. 2 and 3) that Zn^{2+} in high concentrations per se had an inhibitory effect on the *p*-NPPase activity.

The effect on the *p*-NPPase activity of addition of the diphosphonates MDP, Cl_2MDP, and EHDP at pH 10.3 is shown in Fig. 4. At 10 mM diphosphonate concentration, only 15% enzyme activity remained when the pH effects of the diphosphonates were compensated for. No significant difference between the diphosphonates was observed. At a diphosphonate concentration below 10^{-4} M, the *p*-NPPase activity was not affected. At pH 8.8 (Fig. 5) a similar inhibition pattern was observed. Remaining enzyme activity at 10 mM diphosphonate concentration varied between 40% and 52% with EHDP as the most potent inhibitor.

Fig. 6 shows the effect of two different

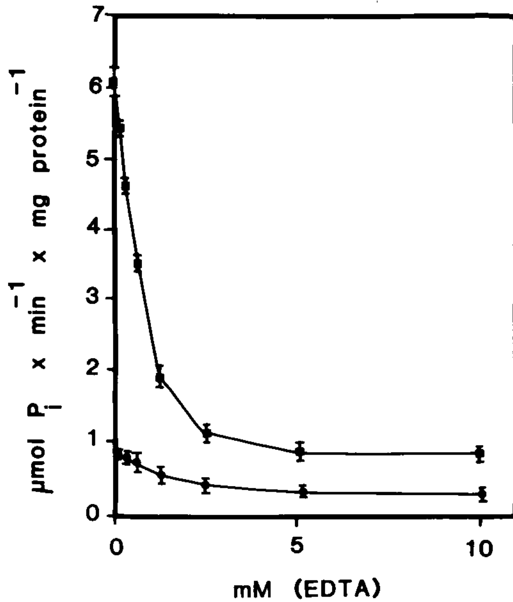

Fig. 1. Influence of EDTA on *p*-NPPase activity in the absence of Mg^{2+} at pH 10.3 (■) and 8.8 (●). Mean values ± SD of five observations.

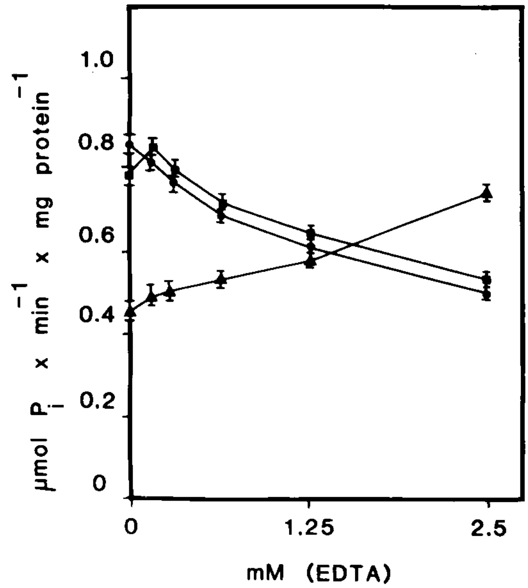

Fig. 3. Influence of Zn^{2+} concentration ((■) 0.15 mM; (▲) 2.5 mM) on *p*-NPPase activity inhibited by EDTA at pH 8.8. (●) No Zn^{2+} added. Mean values ± SD of five observations. Measurements performed in the absence of Mg^{2+}.

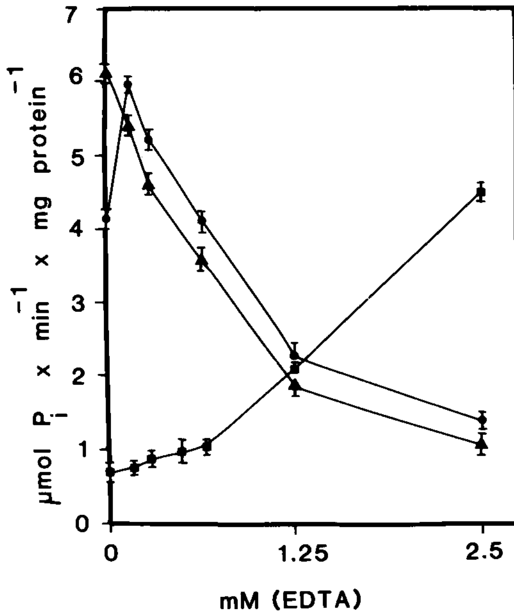

Fig. 2. Influence of Zn^{2+} concentration ((●) 0.15 mM; (■) 2.5 mM) on *p*-NPPase activity inhibited by EDTA at pH 10.3. (▲) No Zn^{2+} added. Mean values ± SD of four to five observations. Measurements performed in the absence of Mg^{2+}.

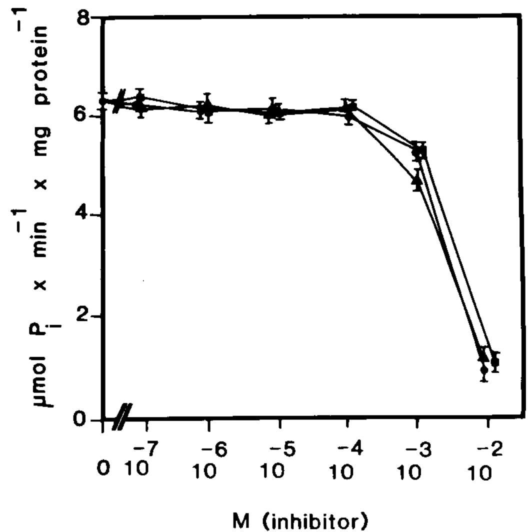

Fig. 4. Effect of diphosphonates—EHDP (▲), Cl_2MDP (■), and MDP (●)—at various concentrations on the *p*-NPPase activity at pH 10.3. Mean values ±SD of five observations. Note that the abscissa is logarithmic.

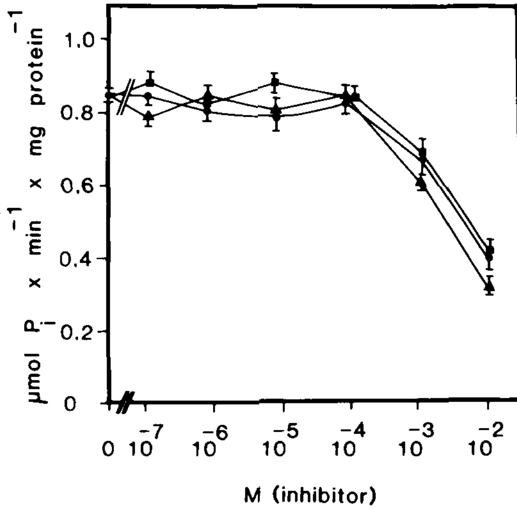

Fig. 5. Effect of diphosphonates—EHDP (▲), Cl_2MDP (■), and MDP (●)—at various concentrations on the *p*-NPPase activity at pH 8.8. Mean values ±SD of four to five observations. Note that the abscissa is logarithmic.

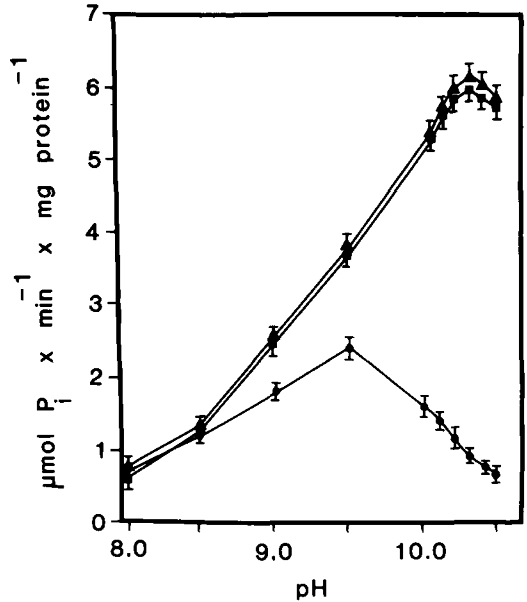

Fig. 7. Influence of pH on inhibition of *p*-NPPase activity by 1 mM L-cysteine (●) and 1 mM L-ascorbic acid (■). (▲) Control. Mean values ± SD of four to five observations.

tetramisoles and their respective D-forms as controls. L-*p*-Bromotetramisole was the most potent inhibitor at concentrations above 10^{-6} M. Maximal inhibitory concentration was found at 10^{-4} M for L-*p*-bromotetramisole and 10^{-3} for levamisole. Concentrations above 10^{-4} M for dexamisole and D-*p*-bromotetramisole had a slightly inhibitory effect.

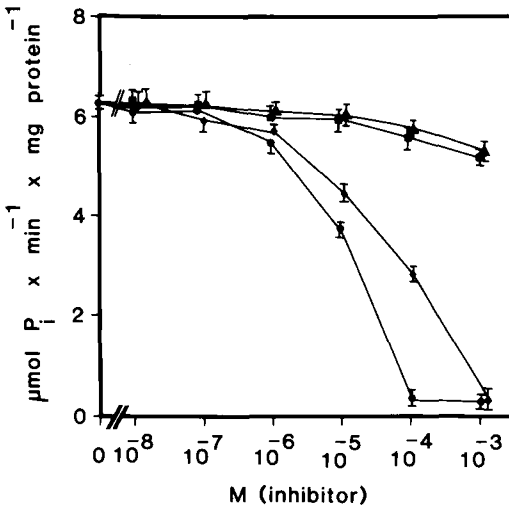

Fig. 6. Effect of L-*p*-bromotetramisole (●), levamisole (◆), D-*p*-bromotetramisole (■), and dexamisole (▲) at various concentrations on the *p*-NPPase activity at pH 10.3. Mean values ± SD of five observations. Note that the abscissa is logarithmic.

An inhibition of the enzyme could also be obtained in the presence of 1 mM L-cysteine (Fig. 7). This inhibition was strongly pH-dependent when *p*-NPP was used as substrate and was maximal at the highest pH (10.5). Addition of 1 mM L-ascorbic acid did not significantly affect the *p*-NPPase activity.

Discussion

The inorganic pyrophosphatase activity (PP_iase) is an important property of non-specific alkaline phosphatase (APase) because there exists considerable evidence that this activity plays a key role in the mineralization process by removing inorganic pyrophosphate (PP_i), a known inhibitor of calcification (6, 7, 11, 13, 22).

Earlier studies of APase from odontoblasts have been performed on whole tissue homogenates (12, 13). By using the microsomal fraction, as in the present study, the specific activity of APase is increased approximately 12-fold (14). Furthermore, contamination of inhibitory and activating substances (such as endogenous Mg^{2+}) is reduced.

In this investigation the effects of different inhibitors on APase were studied at pH 10.3 and 8.8. The former pH is optimal for the APase with *p*-NPP as substrate, and the latter is optimal when PP_i is the substrate (12, 13). The inhibition with EDTA was more pronounced at pH 10.3 than at pH 8.8, probably because of the higher binding capacity of EDTA at pH 10.3 (21).

As could be expected, the inhibiting effect of a chelating agent such as EDTA was eliminated by the addition of Zn^{2+} to the medium. At both pH 8.8 and 10.3 the original enzyme activity could be restored almost completely by the addition of Zn^{2+} in amounts that were equimolar to EDTA in the medium. These findings are in agreement with those of Wöltgens (21) in a study of APase in calcifying hamster molars. It is known that Zn^{2+} is a cofactor for PP_iase (20). The fact that *p*-NPPase and PP_iase activity belong to the same enzyme (10) suggests that the reactivation of *p*-NPPase is a reactivation by its cofactor Zn^{2+}. It is also possible that the added Zn^{2+} formed a complex with EDTA, resulting in a lower inhibition by EDTA.

Diphosphonates are structural analogues of PP_i. They have, like this substance, been shown to inhibit mineralization, probably by an action on the hydroxyapatite crystal surface (9). Another mechanism of action has been proposed by Wöltgens (21). He suggested that the inhibition was the result of complex formation with cofactors of PP_iase such as Zn^{2+} or Co^{2+}. The diphosphonates were able to inhibit the odontoblast *p*-NPPase at both pH 8.8 and 10.3, which agrees with earlier suggestions that diphosphonates act as complexing substances like EDTA (21).

Another type of inhibition was obtained with the tetramisoles. An almost complete inhibition of the *p*-NPPase activity of odontoblasts could be obtained. The strongest inhibitors were L-*p*-bromotetramisole and levamisole, whereas D-*p*-bromotetramisole and dexamisole had a very low inhibiting action. It has been shown that a very low concentration of tetramisole or its analogues is able to inhibit the alkaline phosphatase in liver and bone almost completely in an uncompetitive manner (3, 4).

An inhibition of the *p*-NPPase could also be obtained in the presence of 1 mM L-cysteine that was strongly pH-dependent. This pH effect is probably due to stimulation of the oxidation of L-cysteine at alkaline conditions, causing a reduction of the enzyme, leading to a lower activity. From previous experiments (5) it is known that L-cysteine is also able to inhibit mineralization, which could be explained as a consequence of the reduced APase activity.

L-Ascorbic acid strongly inhibits mineralization in vitro (5), and this effect is maximal at pH 8.5. Unlike the L-cysteine, this effect is evidently not a function of any inhibitory effect on the *p*-NPPase activity. It has been shown that an inhibitory effect of L-ascorbic acid is directed towards the PP_iase activity of hamster molars (23). This effect was explained by a redox reaction between the enzyme and L-ascorbic acid which was stimulated by PP_i but not by *p*-NPP.

Effects of complexing agents such as diphosphonates on cofactors can better be studied with *p*-NPP as substrate because *p*-NPP itself is not a complexing agent, whereas PP_i complexes various divalent cations, introducing thereby a complicating factor. However, PP_i is one substrate that occurs naturally in the body, and, therefore, the effect of the inhibitors on the PP_iase activity probably reflects more the physiological conditions than the effects on *p*-NPPase activity.

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