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THE EFFECT OF CALCIUM IONS ON THE ATPase ACTIVITY OF PIG KIDNEY ALKALINE PHOSPHATASE

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SUMMARY

1. The effect of Ca^{2+} on the ATPase activity of pig kidney alkaline phosphatase has been studied under a number of conditions.

2. Contrary to an earlier report, it was found that Ca^{2+} and Mg^{2+} did not prevent substrate inhibition by ATP at either pH 8.0 or 9.0. At inhibitory levels of ATP however, activity was always greater when Mg^{2+} or Ca^{2+} was present.

3. The effect of Ca^{2+} on ATPase activity was pH-dependent. At pH 8.0, increasing cation concentrations up to 50 mM brought about a doubling in enzymic activity. The inclusion of 1 mM Mg^{2+} together with Ca^{2+} resulted in further activation. At pH 9.0 ATPase was stimulated by Ca^{2+} at concentrations up to 10 mM but higher levels resulted in a fall in activity. The activity at a concentration of 50 mM Ca^{2+} was still greater, however, than with ATP alone. Because Ca^{2+} is inhibitory only at higher pH values, Ca^{2+} appears to lower the pH optimum of the ATPase reaction.

4. The presence of 5 mM Ca^{2+} overcomes the inhibition of ATPase by Mg^{2+} at pH 9.0, but at pH 8.0, Ca^{2+} had little effect on the response of reaction velocity to Mg^{2+} concentration.

5. These findings are discussed in terms of the proposed identity of alkaline phosphatase with a Ca^{2+}-stimulated ATPase important in the transport of Ca^{2+} across biological membranes.

INTRODUCTION

It has been known for some time that the level of alkaline phosphatase (EC 3.1.3.1) in the intestine is decreased in rickets (Pileggi *et al.*[1]) and recent reports have described how in vitamin D-deficient chicks, intestinal brush border alkaline phosphatase activity was increased following the administration of vitamin D_3[2,3]. The increase in alkaline phosphatase was accompanied by a rise in the level of a Ca^{2+}-stimulated ATPase and the time course of the change in ATPase level appeared to be identical with that of alkaline phosphatase. This evidence, together with the now well-established ability of alkaline phosphatase to hydrolyse ATP[4-6] implied that alkaline phosphatase was identical with, or a component of, the Ca^{2+}-stimulated ATPase. Haussler *et al.*[2] and Norman *et al.*[3] also showed that an increase in the transport of Ca^{2+} across isolated segments of chick intestine after the administration of vitamin D_3 occurred in parallel with the rise in alkaline phosphatase and Ca^{2+}-stimul-

ated ATPase levels. It was suggested that the long-sought physiological function of alkaline phosphatase may be connected with the active transport of Ca^{2+} and that it may interact, in some way unknown at present, with a specific Ca^{2+}-binding protein the synthesis of which is also induced by vitamin D_3 (see also Deluca[8] for a review).

We have previously shown[6] that the addition of Mg^{2+} to reaction mixtures containing pig kidney alkaline phosphatase stimulated ATPase activity until the Mg^{2+}/ATP ratio reached unity; further addition of the cation inhibited enzymic activity. We considered that the alternating pattern of stimulation and inhibition dependent on the Mg^{2+}/ATP ratio could be important in regulating bivalent cation reabsorption, should alkaline phosphatase be implicated in such a transport process.

This paper describes a study of the effects of Ca^{2+} and Mg^{2+} on the ATPase activity associated with pig kidney alkaline phosphatase. The results are not easy to interpret in terms of a single cation-stimulated, membrane-localised enzyme, which is directly coupled to ion transport.

EXPERIMENTAL

Materials

The monosodium salt of ATP was obtained from the Sigma (London) Chemical Co. Ltd, London, S W.6., U.K. Tris and $MgSO_4$ (both of analytical reagent grade) were obtained from Fison-Scientific Apparatus Ltd, Loughborough, Leics., U.K. and analytical reagent grade $CaCl_2 \cdot 2H_2O$ from Hopkin and Williams, Ltd, Chadwell Heath, Essex, U.K.

Enzyme

Pig kidney alkaline phosphatase was purified by the methods previously described[9]. The enzyme in 0.05 M Tris–HCl buffer, pH 7.6, was stored at −15 °C until required.

Measurement of ATPase

Fresh solutions of ATP, $MgSO_4$ and $CaCl_2$ in 0.1 M Tris–HCl buffer, pH 9.0, were prepared daily. The pH of the solutions was adjusted to 9.0 if necessary. Buffered reaction mixtures were of 2 ml volume and contained ATP, Mg^{2+} and Ca^{2+} at the concentrations indicated in the section Results and Discussion. The addition of enzyme solution (20 μl) started the reactions which were allowed to continue for up to 10 min at 30 °C. The P_i released was determined by the method of Baginski *et al.*[10] and any interference by high ATP levels was overcome by constructing a series of standard curves for P_i containing appropriate amounts of ATP.

Effects of pH

ATPase activity was assayed at a number of pH values ranging from pH 7.0 to 9.0. The necessary buffers were prepared using the 0.1 M Tris–HCl system. The pH values of the reaction mixtures were determined with a glass microelectrode.

$MgATP^{2-}$ and $CaATP^{2-}$ complexes

The dissociation constants for $MgATP^{2-}$ and $CaATP^{2-}$ complexes are $1.37 \cdot 10^{-5}$

and $3.23 \cdot 10^{-5}$, respectively[11] and it was assumed that when bivalent cation and ATP were present in solution in equal amounts, all the nucleoside triphosphate was complexed as bivalent anion.

RESULTS AND DISCUSSION

In discussing the actions of kidney alkaline phosphatase on ATP and $MgATP^{2-}$, we suggested[6] that although both these forms may be substrates for the enzyme, the $MgATP^{2-}$ complex differed from the free nucleotide triphosphate in that high concentrations of the complex did not result in substrate inhibition. As a result of subsequent experiments, this suggestion needs some amendment.

Fig. 1 shows the effect of increasing concentrations of ATP, $MgATP^{2-}$ and $CaATP^{2-}$ on the initial reaction velocity v at pH 9.0. All three substrates are inhibitory, but at any given substrate concentration the velocity is higher with the complexes than with free ATP. This led to the belief initially that the complexes were non-inhibitory.

The greater activity that is seen with the complexes at any given substrate concentration indicates that the enzyme has been activated. The activation may be brought about by free Mg^{2+} and Ca^{2+}. These are likely to be present in very small amounts however (a 1-mM mixture of Ca^{2+} and ATP is 5 μM with respect to free Ca^{2+}) so that it is possible that the $MgATP^{2-}$ and $CaATP^{2-}$ complexes are also partly stimulatory. The increased activity does not seem to arise from a faster breakdown of $MgATP^{2-}$ relative to ATP for it appears that the complex may be a poorer substrate with a lower V (ref. 6).

The substrate-inhibition studies were repeated at pH 8.0 where it was also found that ATP and both the complexes were inhibitory.

The sample of ATP used in all the experiments contained free phosphate (approx. 1 %) and it was considered that the rising level of P_i with increasing substrate might contribute to the substrate inhibition because P_i is a well-known competitive inhibitor of alkaline phosphatase. Experiments in which the level of P_i in the incubation mixture was kept constant by the addition of suitable amounts of a stock solution showed, however, that the decline in activity with increasing substrate concentration was a true effect of ATP, $MgATP^{2-}$ and $CaATP^{2-}$.

Effect of Ca^{2+} on ATPase activity

The effect of Ca^{2+} on enzymic activity depended on whether Mg^{2+} was also included in the reaction mixtures. Fig. 2 shows results obtained at pH values of 8.0 and 9.0 with an ATP concentration of 1 mM. The addition of Ca^{2+} to ATP causes an initial stimulation of activity similar to that observed on the addition of Mg^{2+} (see also Fig. 3). Higher levels of Ca^{2+} result in a fall of activity but, even when a concentration of 50 mM is reached the activity is still greater than with ATP alone. The degree of activation by Ca^{2+} at the peak of activity was approximately 2-fold. The exact amount of activation varied slightly with different enzyme preparations but was always close to this figure. With $MgATP^{2-}$ as substrate the initial stimulation by Ca^{2+} is not seen and there is progressive inhibition up to 50 mM Ca^{2+}. The enzyme may have been already stimulated maximally by Mg^{2+} or $MgATP^{2-}$ so explaining the absence of the initial activation by Ca^{2+}.

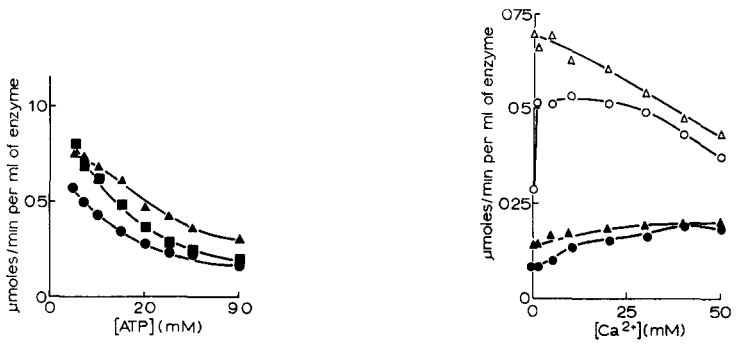

Fig 1. The effect of substrate concentration S, on initial reaction velocity ●, ATP alone, ▲, $MgATP^{2-}$, ■, $CaATP^{2-}$

Fig 2 The effect of Ca^{2+} on initial reaction velocity at pH 9.0 (○, △) and pH 8.0 (●, ▲) ○, ●, ATP alone, △, ▲, $MgATP^{2-}$ The substrate concentration in all experiments was 1 mM.

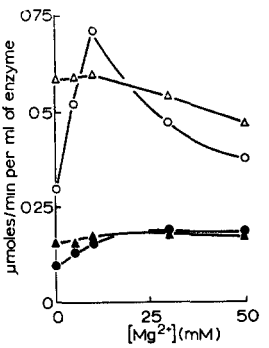

Fig 3. The effect of Mg^{2+} on initial reaction velocity in the presence of Ca^{2+} at pH 9.0 (○, △) and pH 8.0 (●, ▲) ○, ●, ATP alone; △, ▲, ATP + 5 mM Ca^{2+} The concentration of ATP in all experiments was 1 mM.

At pH 8.0 no inhibition by Ca^{2+} could be detected within the range of concentrations tested. With ATP as substrate, 50 mM Ca^{2+} more than doubled the enzymic activity but activities were still low relative to those at pH 9.0. With $MgATP^{2-}$ the increase brought about by 50 mM Ca^{2+} was approx. 50 %. As at pH 9.0 however, the initial activity with $MgATP^{2-}$ was greater than with ATP.

Effect of Ca^{2+} on the inhibition by Mg^{2+} and excess ATP

At pH 9.0 Mg^{2+} activates ATPase activity until the concentration exceeds the ATP concentration when it becomes inhibitory[6]. As can be seen in Fig. 3, the presence of 5 mM Ca^{2+} "smooths out" the *v versus* $[Mg^{2+}]$ curve by hindering the inhibition at higher Mg^{2+} levels. At pH 8.0, Ca^{2+} had little effect on the response of reaction velocity to Mg^{2+} concentration. Substrate inhibition by ATP at pH 9.0 was not decreased by 5 mM Ca^{2+}. Higher concentrations of Ca^{2+} could not be tested because of the precipitation that occurs with 5 mM ATP.

Ca^{2+} effects at different pH values

The difference in the results obtained with experiments at pH 8.0 and 9.0 suggested that the actions of bivalent cations may be very sensitive to pH and the

results of a fuller investigation are shown in Figs 4 and 5. For ATP and $MgATP^{2-}$ at 1 mM there was a steady increase in activity with rising pH. The highest pH tested was still sub-optimal at this substrate concentration. The addition of Ca^{2+} (50 mM) reduced the apparent pH optima by being inhibitory at the higher pH values. Below pH 8.0, however, 5 mM and 50 mM Ca^{2+} had little effect on the pH curves. Similar results were obtained at substrate concentrations of 0.5 mM but the lower level of 5 mM Ca^{2+} was sufficient to show the effects on pH optima.

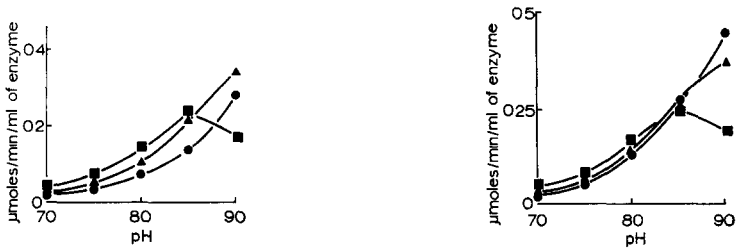

Fig 4. The effect of Ca^{2+} on pH optimum of the ATPase reaction ●, 1 mM ATP, ▲, 1 mM ATP + 5 mM Ca^{2+}; ■, 1 mM ATP + 50 mM Ca^{2+}

Fig 5 The effect of Ca^{2+} on pH optimum of the ATPase reaction when Mg^{2+} is present. ●, 1 mM $MgATP^{2-}$, ▲, 1 mM $MgATP^{2-}$ + 5 mM Ca^{2+}; ■, 1 mM $MgATP^{2-}$ + 50 mM Ca^{2+}

CONCLUSIONS

It is difficult to interpret these observations meaningfully in terms of a Ca^{2+}-stimulated ATPase which might be important in transporting Ca^{2+}. Certainly, Ca^{2+} and Mg^{2+} can be shown to stimulate ATPase activity considerably at relatively high pH values and Ca^{2+} can overcome the inhibitions caused by excess Mg^{2+} but, at pH values more likely to be met *in vivo* the modifying effects of Ca^{2+} and Mg^{2+} seem less pronounced. Although the relative increase in activity brought about by Ca^{2+} or Mg^{2+} is approximately the same at pH 8.0 as it is at pH 9.0 (2-fold) the low activity at pH 8.0 raises doubt perhaps about the importance of alkaline phosphatase as a Ca^{2+}-stimulated ATPase.

It is possible that the enzyme extraction procedure, which involves the use of *n*-butanol, changes the response of the enzyme to numerous factors including Ca^{2+} and that studies carried out on fractions of plasma membrane may approximate more closely to the state of the enzyme *in vivo* and reveal a closer connection between ATPase activity at pH 7–8 and transport of Ca^{2+}. Norman *et al.*[3] worked with preparations of intestinal brush border which should have contained intact enzyme, yet they were able to demonstrate only a 30% stimulation in ATPase activity by the addition of Ca^{2+}. These authors considered, however, that the stimulation was sufficient to account for the increased transport of Ca^{2+}. The pH at which the ATPase activity was determined was not given. Holdsworth[12] concluded that alkaline phosphatase was not directly implicated in the transport of Ca^{2+} on the basis of experiments in which uptake of Ca^{2+} continued unabated following inhibition of the enzyme by L-phenylalanine.

The indefinite evidence linking Ca^{2+} transport with alkaline phosphatase and other reports which associate the enzyme with fat absorption and choline transport[13,14]

point perhaps to the view that the enzyme occupies a relatively non-specific role in several transport processes. Kimmich[15] has put forward a general scheme for membrane transport involving phosphorylated intermediates generated from (Na^+ + Mg^{2+})-activated ATPase. He suggests that a free energy change accompanying the dephosphorylation by different routes of one or more common intermediates may be utilised for the movement across the membrane of many substances including cations, sugars and amino acids for which specific carriers may exist. Alkaline phosphatase may have a dephosphorylating role and in this way partly regulate the flux of materials across the membrane. If such is the case then one might expect to find that the activity of the enzyme is regulated by a variety of compounds, some stimulatory, *e.g.* Ca^{2+}, and others inhibitory, and the finding that L-phenylalanine[16,17] binds to alkaline phosphatase in its phosphorylated form may be important in this connection.

ACKNOWLEDGMENT

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