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STIMULATION BY ATP OF ALKALINE PHOSPHATASE IN PLACENTAL PLASMA MEMBRANES

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Summary

1. ATP stimulated the *p*-nitrophenyl phosphatase activity of placental plasma membranes, with an increase in activity of approximately 100% at 5 mM ATP. The stimulation was not dependent on the presence of Mg^{2+}.

2. The K_m for *p*-nitrophenyl phosphate was not changed by the presence of 5 mM ATP.

3. ATP hydrolysis by the plasma membrane preparation under the same assay conditions as for alkaline phosphatase was not influenced by the presence of 5 mM *p*-nitrophenyl phosphate.

4. Extraction of the plasma membrane preparation with *n*-butanol abolished the stimulatory effect of ATP, as well as Ca^{2+}-activated ATPase activity.

Introduction

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1), a membrane-bound enzyme, is considered to be involved in transport across cell membranes, although its exact role is unknown [1]. ATP acts as a substrate for the enzyme [2,3], and it has been suggested that alkaline phosphatase and (Ca^{2+} + Mg^{2+})-activated ATPase (EC 3.6.1.3) may be the same enzyme [4,5]. In investigating a possible relationship between the two enzymes in placental plasma membranes, we have observed that ATP stimulates *p*-nitrophenyl phosphatase activity. The characteristics of this stimulation by ATP are described in this report.

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Materials and Methods

Plasma membrane preparation. Plasma membranes were isolated as described previously [6] from placentas taken from guinea pigs at 55—60 days of gestation. The final membrane preparation was suspended in 5 mM Tris buffer (pH 7.6) plus 0.5 mM imidazole and 100 mM NaCl, and stored at 4 °C.

Alkaline phosphatase assay. Alkaline phosphatase activity was assayed at pH 10.2, by addition of 50 µl plasma membrane preparation (containing 5—10 µg protein) to a buffer-substrate mixture containing 100 mM bicarbonate/carbonate buffer, 10 mM Mg^{2+} (as $MgCl_2$) and 5 mM *p*-nitrophenyl phosphate. Where appropriate, ATP (disodium salt) was added in the range 0.1—10 mM. Final volume was 0.6 ml. Samples were incubated at 37 °C for 15 min and the reaction was stopped by addition of 2 ml 0.1 M NaOH. Release of *p*-nitrophenol was measured spectrophotometrically at 405 nm. Protein was determined by the method of Lowry et al. [7]. Enzyme activity was expressed as µmol *p*-nitrophenol released per mg protein per h.

ATPase assay. Ca^{2+}-activated ATPase activity was assayed at pH 8.2 in the presence of 5 mM Ca^{2+} as described previously [6].

Purification of alkaline phosphatase. Alkaline phosphatase was further purified by a procedure based on that described by Ghosh and Fishman [8]. The placental plasma membrane preparation was stirred with *n*-butanol (40 ml butanol: 100 ml membrane preparation) for one h at 4 °C and then for 10 min at 37 °C. The emulsion was centrifuged at 14 600 × *g* for 30 min to separate the aqueous phase, which was dialyzed overnight against 50 mM Tris · HCl buffer (pH 8.6). This preparation was subjected to ammonium sulfate fractionation, by precipitating with 90% ammonium sulfate, and extracting the precipitate with solutions of decreasing saturation [8]. The 40—80% saturation fraction was resuspended in 5 ml 50 mM Tris · HCl, pH 8.6 and dialyzed overnight against the same buffer. The enzyme solution was layered onto a 60 cm × 2.5 cm column of Sephadex G-200 (Pharmacia) and eluted with 50 mM Tris · HCl, pH 8.6. 10-ml fractions were assayed for alkaline phosphatase and total protein.

Results

Effect of ATP on alkaline phosphatase activity. The initial *p*-nitrophenyl phosphate phosphatase activity (without ATP) of the placental plasma membrane preparation averaged 4—5 µmol per mg protein per h in the absence of Mg^{2+} and 11—13 µmol per mg protein per h in the presence of 10 mM Mg^{2+}. The Ca^{2+}-ATPase activity ranged from 30—40 µmol P_i released per mg protein per h.

Addition of ATP (disodium salt) to the incubation medium in increasing concentrations (0.1—10 mM) promoted increased hydrolysis of *p*-nitrophenyl phosphate. This occurred both in the absence of Mg^{2+} and when 10 mM Mg^{2+} was present (Fig. 1). A maximal increase in activity of approximately 100% occurred at an ATP concentration of 5 mM.

The effect of ATP on *p*-nitrophenyl phosphate hydrolysis was further examined by incubating the enzyme preparation with increasing concentrations

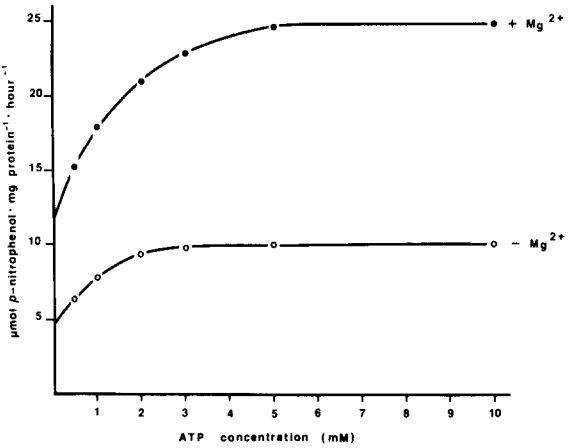

Fig. 1. Stimulation of alkaline phosphatase in intact plasma membranes by ATP. The incubation mixtures consisted of 100 mM bicarbonate/carbonate buffer, pH 10.2, 5 mM *p*-nitrophenyl phosphate, increasing concentrations of ATP, and either 10 mM Mg^{2+} (●———●) or without Mg^{2+} (○———○).

of *p*-nitrophenyl phosphate (0.5—20 mM) either without added ATP or at a constant concentration of 5 mM ATP. The release of *p*-nitrophenol reached a maximum at 10 mM *p*-nitrophenyl phosphate whether or not ATP was present in the incubation medium. The K_m for *p*-nitrophenyl phosphate was 1.44 ± 0.13 mM (mean ± S.E., $n = 4$) in the absence of ATP and 1.32 ± 0.08 mM (mean ± S.E., $n = 5$) in the presence of ATP (Fig. 2). These values are not significantly different.

Hydrolysis of ATP. By measuring the release of inorganic phosphate and *p*-nitrophenol under identical incubation conditions, it is possible to determine the extent of ATP hydrolysis in addition to *p*-nitrophenyl phosphate hydrolysis. Some hydrolysis of ATP would be expected since (Ca^{2+} + Mg^{2+})-activated ATPase of placenta shows partial activity at pH 10.2 [6]. Also, since ATP acts as an alternate substrate for alkaline phosphatase (though with low efficiency)

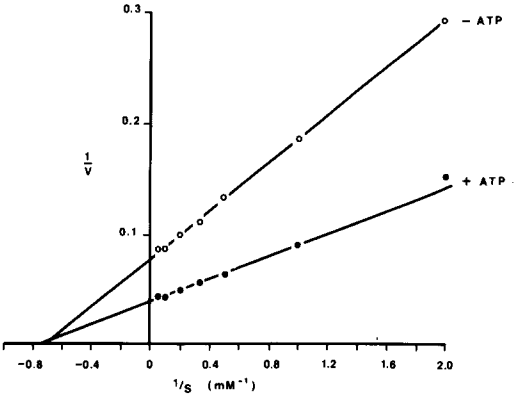

Fig. 2. Lineweaver-Burk plots of alkaline phosphatase activity in the absence (○———○) or presence of ATP (●———●). *V* is expressed in μmol *p*-nitrophenol liberated per mg protein per h, and [*S*] in mM.

TABLE I

PHOSPHATE AND *p*-NITROPHENOL RELEASE FROM *p*-NITROPHENYL PHOSPHATE IN THE ABSENCE OR PRESENCE OF ATP

All incubations were performed at pH 10.2 in the presence of 10 mM Mg^{2+}, and *p*-nitrophenyl phosphate and ATP concentrations were 5 mM. Phosphate and *p*-nitrophenol release are expressed as μmol per mg protein per h.

	P_i	*p*-Nitrophenol
p-Nitrophenyl phosphate alone	11.8	11.4
p-Nitrophenyl phosphate plus ATP	29.1	22.1
ATP alone	6.5	—

[2,3], it is important to determine the effect of *p*-nitrophenyl phosphate on ATP hydrolysis as well as the reverse. The release of P_i from *p*-nitrophenyl phosphate alone (5 mM), ATP alone (5 mM) and equimolar ATP plus *p*-nitrophenyl phosphate, and the release of *p*-nitrophenol from *p*-nitrophenyl phosphate alone and in the presence of 5 mM ATP were measured at pH 10.2 (Table I).

There was good agreement between the release of P_i and *p*-nitrophenol from *p*-nitrophenyl phosphate alone (respectively 11.8 and 11.4 μmol per mg protein per h). The release of P_i from ATP alone at pH 10.2 (6.5 μmol per mg protein per h) was in the range predicted from the pH curves of Shami and Radde [6]. ATP stimulated *p*-nitrophenol release by 94%. Since P_i release from *p*-nitrophenyl phosphate must be enhanced to the same extent, the net P_i release from ATP in the presence of 5 mM *p*-nitrophenyl phosphate was calculated to be 6.2 μmol per mg protein per h. Thus inhibition of ATP hydrolysis by *p*-nitrophenyl phosphate was less than 5%, and is considered to be within the experimental error of the method.

Effect of ATP on purified alkaline phosphatase. The placental plasma membrane preparation was subjected to butanol extraction, ammonium sulfate precipitation and gel filtration. The elution profile of alkaline phosphatase on Sephadex G-200 is shown in Fig. 3. The peak of alkaline phosphatase activity

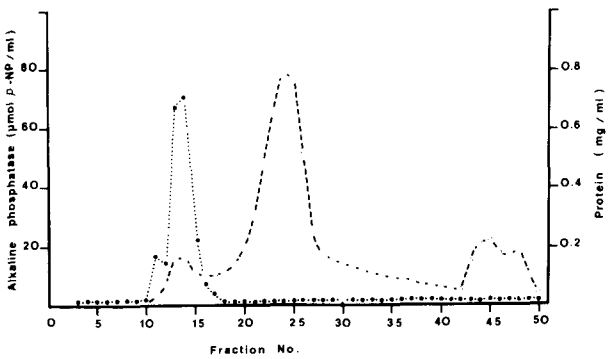

Fig. 3. Elution profile of alkaline phosphatase and total protein on Sephadex G-200. The eluent consisted of 50 mM Tris · HCl, pH 8.6; fraction volume was 10 ml. Alkaline phosphatase activity (μmol *p*-nitrophenol liberated per ml eluate per h): ○———○; protein (absorbance at 280 nm): ●———●.

(fractions 11—16) corresponds to the Variant B described by Ghosh and Fishman [8] for human placental alkaline phosphatase. The specific activity of pooled fractions 11—16 was 520 μmol *p*-nitrophenol liberated per mg protein per h, representing a 45-fold purification of the enzyme compared to the activity in the plasma membrane preparation. The K_m for *p*-nitrophenyl phosphate with the purified enzyme was 1.39 ± 0.04 (mean ± S.E., $n = 3$), which was not significantly different from the values derived using the intact plasma membrane preparation.

Stimulation of *p*-nitrophenyl phosphate hydrolysis by addition of ATP was not observed with the purified alkaline phosphatase preparation, and in fact a slight inhibition (5—8%) occurred at a concentration of 5 mM ATP. Ca^{2+}-dependent ATPase activity was also abolished by the purification procedure. A similar degree of inhibition by ATP was found using the butanol-extracted preparation, and Ca^{2+}-ATPase activity was also lost in this first step in the purification procedure. Thus both the stimulatory effect of ATP on alkaline phosphatase and the Ca^{2+}-ATPase activity appear to require intact plasma membranes.

Discussion

Alkaline phosphatase activity in intact placental plasma membranes was stimulated approximately 100% by 5 mM ATP. A similar effect has been described for plasma membranes of bone cells [9]. Stimulation by ATP did not require Mg^{2+}, and the percentage stimulation was unchanged in the presence and absence of Mg^{2+}. However, enzyme activity was much lower in the absence of Mg^{2+}.

In addition to the stimulatory effect of ATP on *p*-nitrophenyl phosphate hydrolysis, ATP was itself consumed during the reaction. ATP is an alternate substrate for alkaline phosphatase, although the rate of ATP hydrolysis is generally much lower (5—15%) than that of phosphomonoesters [10—12]. The hydrolysis of ATP is better accounted for by the (Ca^{2+} + Mg^{2+})-activated ATPase of the plasma membrane preparation. The extent of hydrolysis at pH 10.2 and 10 mM Mg^{2+} is in the range predicted from previous studies of ATPase activity [6], indicating only a small contribution from alkaline phosphatase. The presence of equimolar *p*-nitrophenyl phosphate exerted very little effect on ATP hydrolysis. Rega et al. [13] reported an inhibitory effect of *p*-nitrophenyl phosphate on ATP hydrolysis by erythrocyte membranes, but a 30-fold excess of *p*-nitrophenyl phosphate was necessary to inhibit ATP hydrolysis by 20%.

ATP did not alter the K_m for *p*-nitrophenyl phosphate, suggesting that ATP exerts its effect at a site other than the active site for *p*-nitrophenyl phosphate hydrolysis. It is possible that ATP binds at the active center for Ca^{2+} (Mg^{2+})-ATPase [13]. If so, a close relationship between the two enzymic activities is postulated. In this regard, ATP was hydrolyzed at the same time that *p*-nitrophenyl phosphate was hydrolyzed, and extraction of the plasma membrane preparation with *n*-butanol abolished both Ca^{2+} (Mg^{2+})-ATPase activity and the stimulatory effect of ATP on alkaline phosphatase. Maximal stimulation of *p*-nitrophenyl phosphatase activity occurred at the same concentration

of ATP (5 mM) as is required for optimal ATPase activity [6]. However, the substrate for Ca^{2+} (Mg^{2+})-ATPase of placenta is believed to be a divalent cation/ATP complex [6], whereas the stimulation of alkaline phosphatase by ATP did not require the presence of divalent cation.

Extraction of lipids from the placental plasma membranes using *n*-butanol abolished the Ca^{2+} (Mg^{2+})-ATPase activity of the preparation. The precise role of the lipid in ATPase activity is not known; possibly the lipoprotein association maintains the protein conformation necessary for enzyme activity. Other mammalian ATPases, including (Na^+ + K^+)-ATPase and the Ca^{2+}-ATPase of sarcoplasmic reticulum, also require lipid factors for activity [14,15]. By contrast, the alkaline phosphatase activity of the placental preparation increased approx. 5-fold following butanol extraction.

A phosphatase activity dependent on the presence of ATP and Ca^{2+} is considered to be a part of the reaction pathway of the (Ca^{2+} + Mg^{2+})-dependent ATPase system of the erythrocyte [13]. Phosphatase activity is also associated with the (Na^+ + K^+)-ATPase of several tissues [16—18] and with the Ca^{2+}-ATPase of sarcoplasmic reticulum [19]. In these cases, the optimal pH for *p*-nitrophenyl phosphate hydrolysis is close to that for ATP hydrolysis (near neutrality). It is not certain from the present study whether a comparable role can be assigned to alkaline phosphatase in placental plasma membranes, since the optimal pH under the assay conditions used (10.2) is quite distinct from that for Ca^{2+} (Mg^{2+})-ATPase (pH 8.2). However, these differences may not prevail in vivo, since the optimum pH for alkaline phosphatase in vitro is strongly dependent on the nature and concentration of both substrate and buffer, and on total ionic strength [1]. Despite these uncertainties, a close relationship between the two enzymes is suggested.

Acknowledgements

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