ATP(Mg^{2+}) INDUCED INHIBITION OF CYCLIC AMP REACTIVITY WITH A SKELETAL MUSCLE PROTEIN KINASE†

Mari K. Haddox, Nancy E. Newton‡, Diane K. Hartle and Nelson D. Goldberg

Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

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SUMMARY: Exposure of a skeletal muscle protein kinase (peak I DEAE fraction) to μmolar ATP in the presence of Mg^{2+} before initiating the binding reaction or the cAMP activable phosphotransferase reaction with cAMP increases the requirement for this cyclic nucleotide in both events about 10 fold. The binding of ATP(Mg^{2+}) to a protein in the kinase preparation was shown by Millipore and Sephadex filtration to parallel the inhibitory effect of the triphosphate. The value of Ks for ATP was in the 10^{-7} M range and 50% inhibition of cAMP (10^{-8} M) binding occurred at approximately 2 x 10^{-7} M ATP. ATP binding activity was associated with protein peaks exhibiting both cAMP binding and phosphotransferase activity after subjecting the kinase (holoenzyme) to sedimentation in sucrose density gradients or electrophoresis by electrofocusing.

In a previous communication (1) we reported that prior incubation (0°, 30 min), of skeletal muscle protein kinase with micromolar concentrations of ATP in the presence of Mg^{2+} (2 mM) dramatically inhibited (75%) the binding of [^{3}H]cAMP (10^{-9} to 10^{-8} M) to the enzyme. ATP or Mg^{2+} alone had no effect. ATP(Mg^{2+}) treatment of the kinase is shown in Fig. 1A to result in an increase in the apparent Ks value (from 2 x 10^{-8} to 2 x 10^{-7} M) for cAMP in the binding reaction and (Fig. 1B) an increase in the requirement for cAMP (from 3 x 10^{-9} M to 5 x 10^{-8} M) as an activator of kinase activity. The apparent difference in the absolute values of Ks and Ka may stem from the different conditions employed in the binding and phosphotransferase experiments or indicate that kinase binding and activation by cAMP may not be absolutely parallel processes.

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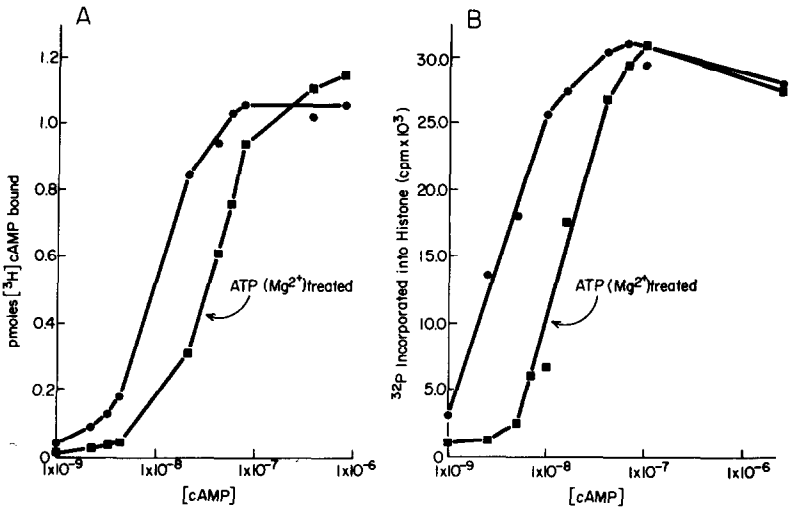

Fig. 1 The effect of prior treatment with ATP(Mg^{2+}) on cAMP binding and activation of protein kinase. A. Pretreatment with ATP(Mg^{2+}) was carried out for 30 min at 0° in a media containing 50 mM Tris·HCl, pH 7.5, 15 µg of protein kinase as prepared previously (6) and when indicated 3 µM ATP and 2 mM $MgCl_2$ in a total volume of 50 µl. After the addition of [^{3}H]cAMP [24 Ci/mMole (Schwartz Bioresearch)], then incubation at 0°, the reaction mixture was passed through a Millipore filter and [^{3}H]cAMP binding determined as previously described (4). B. The protein kinase was preincubated for 30 min at 0° in a reaction mixture containing 3 µM ATP, 2 mM $MgCl_2$, 50 mM Tris·HCl, pH 7.5, 10 mM KF and 0.3 mM EGTA in a total volume of 100 µl, followed by the addition of cAMP for 30 min at 0°. The phosphotransferase reaction was initiated by the addition of histone and 1 million cpm [γ^{32}P]ATP to achieve final concentrations of 0.2 mg/ml and 0.1 mM, respectively. After incubation for 10 min at 20°, ^{32}P incorporation into histone was determined as previously described (5).

In any case, the effect of exposing the enzyme to ATP(Mg^{2+}) was to increase the requirement for the cyclic nucleotide approximately 10 fold in both the binding and activation processes.

It was found that the nucleotide induced change in kinase affinity for cAMP was specific for ATP; other nucleotide polyphosphates, phosphohexoses, triosephosphates, inorganic phosphate or pyrophosphate had little or no effect.

Millipore filtration of reaction mixtures containing [^{3}H]ATP(Mg^{2+}) and the DEAE peak I protein kinase (after interaction for 30 min at 0°) resulted in the retention of a [^{3}H]nucleotide-protein complex on the cellulose ester membranes. At least 95% of the acid extractable [^{3}H]nucleotide in the

complexes retained by the filters co-chromatographed on polyethylenimine cellulose thin layer plates with authentic ATP. Complexes resulting from the interaction of [^{3}H]ATP or [γ^{32}P]ATP with protein kinase, which were recovered in the exclusion peaks from Sephadex G-25 columns, were also found to contain an isotopically labeled component which was identified chromatographically on polyethylenimine cellulose to be (>95%) ATP.

Fig. 2 shows the results of experiments designed to determine if the inhibitory effect of the triphosphate was related to the observed binding of ATP. A progressive inhibition of [^{3}H]cAMP (1×10^{-8} M) binding occurred with concentrations of the triphosphate (unlabeled) from 2.5×10^{-9} M to 5×10^{-7} M and in a parallel experiment a correspondingly greater amount of [^{3}H]ATP was

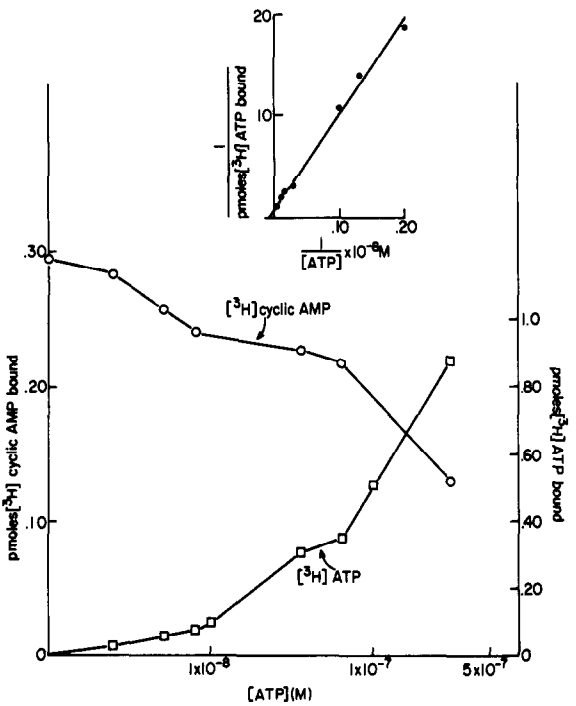

Fig. 2 Relation between kinase complexing with ATP or [^{3}H]ATP and the reduction in [^{3}H]cAMP binding to the kinase. Pretreatment of the kinase was conducted as described in Fig. 1 with either ATP or [^{3}H]ATP, followed by incubation (30 min, 0°) with either [^{3}H]cAMP in reactions containing unlabeled ATP, or unlabeled cyclic nucleotide in those with [^{3}H]ATP. The binding of [^{3}H]cAMP or [^{3}H]ATP was determined by the Millipore filter retention method (4).

shown to complex with protein as the concentration of the triphosphate was increased. The results of these experiments indicate that the binding of [^{3}H]ATP and the inhibitory effects of the triphosphate on [^{3}H]cAMP reactivity with the kinase are proportional in the range of ATP concentrations tested. A 50% reduction in [^{3}H]cAMP (10^{-8} M) binding occurred at a concentration of 2.5×10^{-7} M ATP. The apparent Ks value for [^{3}H]ATP was determined to be 1.3×10^{-7} M (inset).

The relationship between ATP binding and the inhibitory effect on protein kinase interaction with cAMP was further established in the following way. Equal aliquots of the kinase preparation previously incubated with or without ATP(Mg^{2+}) were chromatographed on Sephadex G-50 (with 1 mM EDTA in the eluting buffer) and the binding of [^{3}H]cAMP determined in fractions representing the exclusion peak (i.e. essentially free of unbound ATP and Mg^{2+}). As shown in Fig. 3B the ATP(Mg^{2+}) treated kinase free of unbound triphosphate and in the presence of sufficient EDTA to prevent any effect of dissociated ATP(Mg^{2+}) bound less than 30% of the [^{3}H]cAMP bound by the nontreated enzyme. In a parallel study conducted with [^{3}H]ATP (in place of the unlabeled triphosphate) (Fig. 3A) it could be demonstrated that radioactive nucleotide apparently complexed with protein was present in the exclusion peak. The total radioactivity present in those fractions was retained on cellulose ester membranes upon filtration (not shown). However, upon reexamining the pooled exclusion peak fractions for cAMP binding capacity and radioactive triphosphate bound after storage for 24 hrs at 0^o in the presence of 1 mM EDTA it was found that no bound [^{3}H]ATP was detectable and that the inhibitory effect on cAMP binding in the peak from the ATP(Mg^{2+}) pretreated enzyme had also disappeared.

It was also determined in the subsequent experiments (not shown) that cAMP does not exchange with kinase-complexed [^{3}H]ATP while the latter readily exchanges with unlabeled ATP. This indicates that separate binding sites exist for the cyclic nucleotide and the triphosphate.

To determine if the ATP(Mg^{2+}) binding protein was indeed associated with

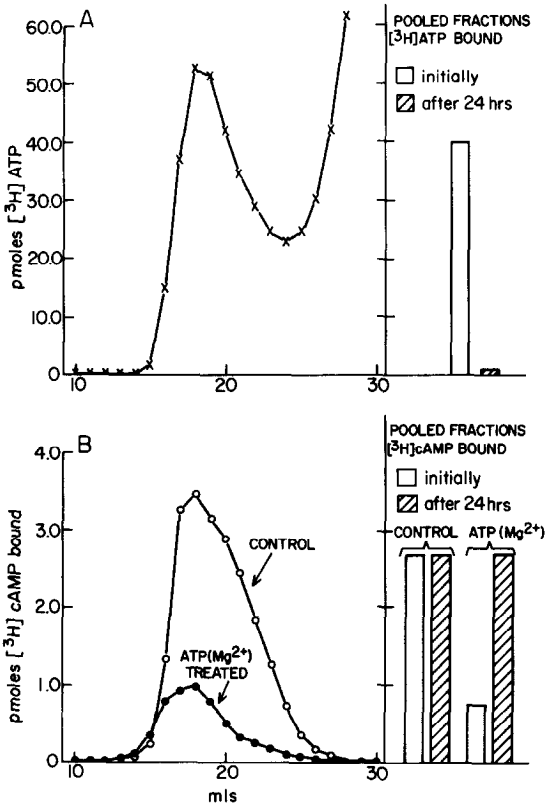

Fig. 3 Reduced cAMP binding capacity of ATP(Mg^{2+}) complexed kinase freed of unbound ATP and Mg^{2+} by Sephadex filtration. After incubation with or without ATP(Mg^{2+}) or [^{3}H]ATP(Mg^{2+}) the reaction mixture (described in Fig. 1) was passed over a Sephadex G-50 column (1.8 x 11 cm) equilibrated and eluted with 25 mM Tris·HCl containing 1 mM EDTA. A. (Left) elution pattern of [^{3}H]ATP from Sephadex G-50; (right) [^{3}H]ATP binding as determined by Millipore filtration in aliquots of pooled fractions 16 through 20 immediately after collection (i.e., "initial") and after storage for 24 hrs at 0° in the presence of 1 mM EDTA. B. (Left) [^{3}H]cAMP binding (Millipore filtration) by fractions of ATP(Mg^{2+}) treated or nontreated ("control") protein kinase eluted from Sephadex G-50 column; (right) [^{3}H]cAMP binding as determined by Millipore filtration by aliquots of pooled fractions 15 through 20 immediately after collection (i.e., "initial") and after 24 hrs of storage at 0° in the presence of 1 mM EDTA.

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the kinase (i.e. holoenzyme) the enzyme was subjected to electrophoresis by electrofocusing over pH gradients of 3 to 10 and 3 to 6. As shown in Fig. 4 the fractions exhibiting cAMP binding as well as catalytic activities also possessed ATP binding activity. The isoelectric point for all three was very close to pH 5.1 in both the broad and narrow pH gradient experiments. A

second ATP binding peak with an isoelectric point of 6.0 (Fig. 4B) was also detected.

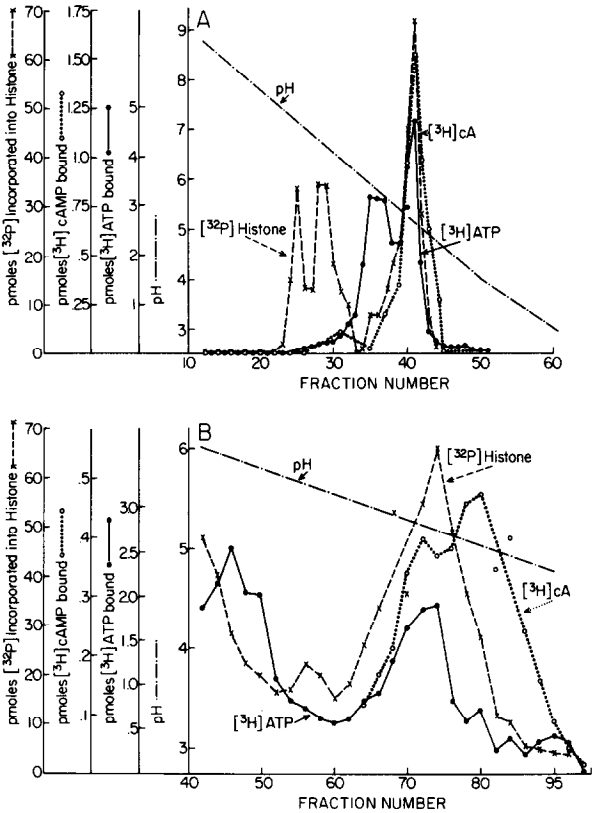

Fig. 4 Electrofocusing of protein kinase. Electrophoresis of the protein kinase (8 µg) was carried out for 48 hrs at a maximum current of 500 v in a 0-50% sucrose gradient (110 ml) containing 1% LKB ampholytes pH 3 to 10 (A) or pH 3 to 6 (B) using an LKB type 8101 electrofocusing column. Serial fractions (1 ml) were collected and pH, cAMP binding, ATP binding and phosphotransferase activity (in the presence of 10^{-6} M cAMP) determined in an aliquot of each fraction (see Figs. 1 and 2 for assay procedures).

The electrofocusing procedure resolved two additional peaks (i.e. pH 7.4 and 7.0) possessing catalytic activity which probably contain catalytic subunits essentially free of regulatory protein subunit since they are almost equally active when assayed in the absence or presence of cAMP. Furthermore, with the narrower pH gradient a second peak of cAMP binding activity with an isoelectric point of 5.0 was also detected.

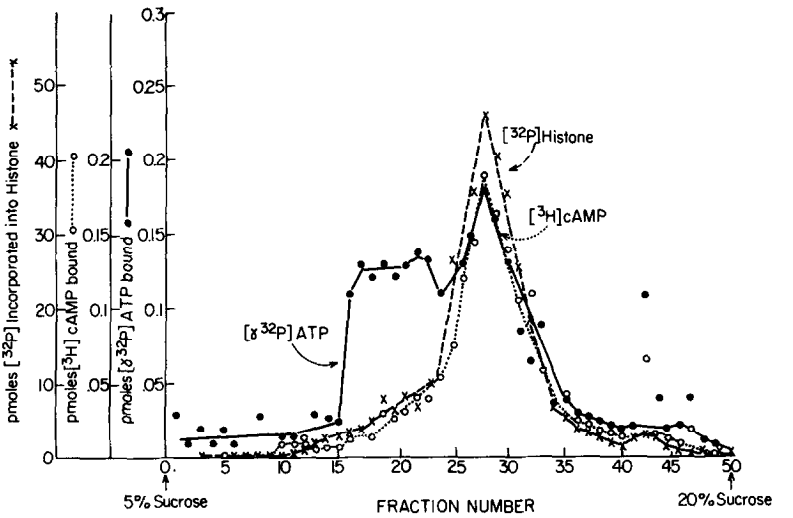

Fig. 5 Sedimentation of protein kinase on a sucrose gradient. A 150 µl sample of the protein kinase was layered onto a 5-20% sucrose gradient containing 50 mM Tris·HCl (pH 7.5) and 1 mM EDTA. Centrifugation was carried out at 2-4° for 20 hrs using a Spinco SW39 rotor at a speed of 39,000 rpm. Serial fractions (2 drops each) were collected by means of continuous upward displacement. Aliquots of each fraction were tested for cAMP and ATP binding capacity and for phosphotransferase activity (in the presence of 10^{-6} M cAMP) using conditions described in Figs. 1 and 2.

The kinase preparation was also subjected to sedimentation in a sucrose density gradient (5 to 20%) and the fractions collected were examined for ATP and cAMP binding activity as well as for phosphotransferase activity (in the presence of cAMP). Fig. 5 shows that there was coincident sedimentation of the component(s) exhibiting each of these properties. A plateau of ATP binding activity was also detected following these peaks.

An ATP binding protein can, therefore, be shown on the basis of both mass and net charge to be associated with this protein kinase. The results are consistent with the hypothesis that ATP binds to a protein which is either closely associated with or an integral part of the holoenzyme (e.g. kinase). The additional ATP binding protein of dissimilar mass and charge may represent another ATP binding protein or one that dissociates from the enzyme during the experimental procedures employed.

DISCUSSION

The evidence presented in this report clearly establishes that the activation of a protein kinase by cyclic AMP may be influenced by another cellular metabolite, ATP. This phenomenon could be viewed as evidence in support of the possibility that a facilitation or suppression of protein kinase activity may be brought about by a mechanism not necessarily involving an alteration in tissue cyclic AMP concentration.

Because tissue concentrations of the triphosphate (i.e. 10^{-3} M) are several magnitudes of order greater than the apparent Ks value determined for ATP with this particular enzyme it would seem that other factors may influence the reactivity of the enzyme with ATP or that the triphosphate may be associated with the enzyme in its natural state. The latter may serve to explain at least in part the discrepancy that exists between the extremely low requirement (i.e. 10^{-9} to 10^{-8} M range) for cAMP with purified protein kinases and the concentrations in the 10^{-7} to 10^{-6} M range of cAMP that exist in most mammalian tissues.

Although an ATP binding moiety appears to be associated with the holoenzyme it is not possible to determine from the evidence presented here whether the highly reactive ATP site is associated with the catalytic or regulatory subunit, a separate as yet unidentified subunit of the kinase or another protein which can interact with the holoenzyme under the conditions employed. Whichever the case it would appear that the binding of ATP to this component affects cAMP interaction with the enzyme.

The relatively great stability of the complex between ATP and the binding component (i.e. stable to filtration on Millipore filters and Sephadex columns) is in itself a feature of the interaction which, to the knowledge of the authors, has not yet been described for any other mammalian enzyme, but is consistent with the unusually low dissociation constant for ATP (i.e. 10^{-7} M). The latter phenomenon would argue against the possibility that the substrate site for ATP on the catalytic subunit is the ATP reactive component involved

in this phenomenon since the Km value for ATP in the phosphotransferase reaction has been determined to be 2 orders of magnitude greater (2).

The original description (1) and the evidence presented here of an ATP(Mg^{2+}) induced impairment of cAMP binding and activation of the kinase is consistent with the recent report by Brostrom et al. (3) that the addition of ATP(Mg^{2+}) promotes the dissociation of [^{3}H]cAMP from the regulatory subunit of the kinase in the presence of catalytic subunit.

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