Activation of erythrocyte Ca^{2+} -plus- Mg^{2+} -stimulated adenosine triphosphatase by protein kinase (cyclic AMP-dependent) inhibitor

Comparison with calmodulin

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Purified protein kinase (cyclic AMP-dependent) inhibitor (PKI) from bovine heart stimulated Ca^{2+} + Mg^{2+} -stimulated ATPase activity in human erythrocytes, the stimulation being maximal at $2\mu\text{g}/0.6\text{ml}$. By contrast, PKI from rabbit skeletal muscle had no effect. Bovine heart PKI stimulated Ca^{2+} + Mg^{2+} -stimulated ATPase by increasing the Ca^{2+} -sensitivity of the enzyme. This contrasted with the stimulation by calmodulin, which increased the maximum velocity of the Ca^{2+} + Mg^{2+} -dependent ATPase in addition to its effect on the Ca^{2+} -sensitivity. Both membrane-bound and Triton X-100-solubilized Ca^{2+} + Mg^{2+} -stimulated ATPase activities were stimulated by PKI, indicating that the stimulation did not require an intact membrane structure. At low Ca^{2+} concentration the stimulation by PKI and saturating concentrations of calmodulin were additive, suggesting that the two effectors acted by distinct mechanisms. Although $5\mu\text{M}$ -cyclic AMP inhibited Ca^{2+} + Mg^{2+} -stimulated ATPase activity by about 20% when measured at low ATP concentrations, probably by stimulation of phosphorylation by an endogenous protein kinase, the stimulation by PKI (about 100%) was not solely due to its antagonism of the protein kinase. This interpretation was supported by a number of observations. First, modification of arginine residues of bovine heart PKI abolished its inhibition of cyclic AMP-dependent protein kinase, but had no effect on the stimulation of Ca^{2+} + Mg^{2+} -stimulated ATPase. Secondly, trifluoperazine ($20\mu\text{M}$) antagonized the stimulation of Ca^{2+} + Mg^{2+} -dependent ATPase by PKI, similarly to its antagonism of calmodulin stimulation, but it did not affect the inhibition of protein kinase by PKI. We conclude that different mechanisms are involved in the inhibition of protein kinase and the stimulation of Ca^{2+} + Mg^{2+} -stimulated ATPase by PKI.

In a number of tissues active Ca^{2+} transport is stimulated on phosphorylation of membrane proteins by cyclic AMP-dependent protein kinase (Nimmo & Cohen, 1977). In the erythrocyte plasma membrane, cyclic AMP has been shown under certain conditions to inhibit $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase (the biochemical expression of the Ca^{2+} pump), probably via an endogenous cyclic AMP-dependent protein kinase (Varghese & Cunningham, 1980). To study further the role of cyclic AMP-dependent

protein kinase on the activity of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, we have investigated the effect of protein (cyclic AMP-dependent) kinase inhibitors (PKI) on the kinetics of Ca^{2+} activation of the Ca^{2+} -transport ATPase. PKI from bovine heart was shown to stimulate $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, more than expected from antagonism of the inhibition of protein kinase. Calmodulin has been shown to be a potent activator of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977) and to regulate cyclic AMP metabolism (Cheung *et al.*, 1978; Cheung, 1980). Since both PKI and calmodulin are heat-stable acidic proteins (Whitehouse *et al.*, 1980; Cheung, 1980), the mechanism of the effects of PKI on the erythrocyte $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase was

Abbreviations used: PKI, protein kinase (cyclic AMP-dependent) inhibitor; $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, Ca^{2+} + Mg^{2+} -stimulated adenosine triphosphatase.

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investigated in further detail and compared with the activation of this enzyme by calmodulin.

Experimental

Materials

Histone type II-A, cyclic AMP-dependent protein kinase, protein kinase catalytic (C) subunit and PKI (all from bovine heart), PKI from rabbit skeletal muscle, calmodulin-depleted phosphodiesterase, ATP (Tris salt), EGTA, EDTA, cyclic AMP, bovine serum albumin, sodium tungstate, imidazole (grade I), maleic acid and boric acid were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DE-52 DEAE-cellulose anion-exchange resin was purchased from Whatman (Maidstone, Kent, U.K.). Trichloroacetic acid and cyclohexane-1,2-dione were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Trifluoperazine {2-trifluoromethyl-10-[3-(4-methylpiperazin-1-yl)-propyl]phenothiazine} hydrochloride was supplied by Smith, Kline and French Co. (Montreal, Quebec, Canada). Calmodulin from bovine brain was a generous gift from Dr. J. H. Wang (University of Manitoba, Winnipeg, Manitoba, Canada). All other salts were analytical reagent grade or the purest grade available.

Membrane isolation and assay

Human erythrocyte 'ghosts' were prepared by the method of Dodge *et al.* (1963) and Roufogalis & Mauldin (1980). Membranes were depleted of endogenous activators, including calmodulin, by treatment of erythrocyte 'ghosts' with 0.1 mM-EDTA/1 mM-Tris/maleate buffer, pH 8.2, as described previously (Roufogalis & Mauldin, 1980). ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase activity was measured in a medium containing (final volume 0.6 ml) 55 mM-Tris/maleate buffer, 66 mM-NaCl, 0.1 mM-ouabain, 6.5 mM-MgCl₂, 0.1 mM-EGTA, 50 μ l of membrane preparation (approx. 0.2 mg of protein) or 50 μ l of Triton X-100-solubilized enzyme (approx. 0.018 mg of protein) and various amounts of CaCl₂ at pH 7.2 as described previously (Roufogalis & Mauldin, 1980). ATP (Tris salt) was added to a final concentration of 2 mM and the reaction conducted for 60 min at 37°C. In experiments designed to measure the influence of cyclic AMP-dependent protein kinase (see Fig. 4) the ATP concentration was decreased to 20 μ M and the incubation time was shortened to 3 min, to maintain the linearity of the ATPase-catalysed hydrolysis. Free Ca^{2+} concentrations are plotted throughout, and were calculated from the Ca^{2+} -EGTA, Ca^{2+} -ATP and Mg^{2+} -ATP binding constants and checked by a Ca^{2+} -selective electrode (Philips IS 561), as described previously (Al-Jobore & Roufogalis, 1981). Calmodulin or protein kinase inhibitors were added to the in-

cubation medium 10 min before the reaction was started with the addition of ATP. Typical results from at least three similar experiments on different membrane preparations are shown. ATPase activity in the absence of added Ca^{2+} was subtracted from total ATPase activity to determine the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase activity.

Assay of phosphodiesterase activity

Phosphodiesterase activity was determined by the method of Sharma *et al.* (1978) using an inorganic phosphate assay. P_i released was measured by an automated Fiske & Subbarow (1925) assay, as described by Raess & Vincenzi (1980).

Assay of protein kinase activity

Inhibition of cyclic AMP-dependent protein kinase activity by PKI was determined with either protein kinase and cyclic AMP or with free catalytic subunit. The reaction mixture contained 24 mg of histone/ml in 50 mM-Mes (4-morpholine-ethanesulphonic acid)/NaOH buffer, pH 6.9, 5 μ M-cyclic AMP, 5 μ g of cyclic AMP-dependent protein kinase (or 5 μ g of cyclic AMP-dependent protein kinase catalytic subunit), 6.5 mM-MgCl₂, 0.1 mM-EGTA, 5 mM-dithiothreitol and [γ -³²P]ATP (100 d.p.m./pmol) at a final ATP concentration of 100 μ M in a total volume of 200 μ l. The reaction mixture was incubated for 10 min in a shaking water bath at 30°C. The reaction was terminated by the addition of 4 ml of ice-cold 5% (w/v) trichloroacetic acid/0.25% sodium tungstate, pH 2, followed immediately by 0.2 ml of 0.63% bovine serum albumin as a carrier protein. The mixture was kept for 5 min in ice and centrifuged for 15 min. The supernatant was carefully removed by aspiration and the precipitate was dissolved in 0.1 ml of 1 M-NaOH, followed by 2 ml of 5% trichloroacetic acid/0.25% sodium tungstate, pH 2. The protein was re-precipitated by adding 0.55 M-H₂SO₄. The precipitate was washed two more times as above and finally dissolved in 0.1 ml of 1 M-NaOH. The radioactivity in this sample was measured in a liquid-scintillation counter.

Purification of PKI

The PKI from bovine heart obtained from Sigma Chemical Co., although reported to be free of 'heat-stable modulator' (calmodulin), was found to contain small but significant contamination by calmodulin, as determined by the phosphodiesterase assay and by radioimmunoassay. The PKI preparation was purified on a Whatman DE-52 DEAE-cellulose column. The PKI preparation from Sigma (5 mg/5 ml) was dialysed against 10 mM-imidazole/HCl buffer, pH 6.8, and applied to a DE-52 DEAE-cellulose column (2.1 cm $\times$ 20 cm) previously equilibrated in the same buffer. The column was washed with 25 ml of the buffer and

eluted with 200 ml of a linear gradient of 10–300 mM-NaCl in 10 mM-imidazole/HCl buffer, pH 6.8. Fractions of volume 2–3 ml were collected. The fractions containing the PKI (as determined by their inhibition of the phosphorylation of histone by the protein kinase catalytic subunit) were pooled (usually 15 ml) and concentrated by dialysis (M_r cut-off 3000) against poly(ethylene glycol). The protein solution, concentrated to approx. 5 ml, was then dialysed against 10 mM-imidazole buffer containing 5 mM-EDTA, followed by extensive dialysis against imidazole buffer to remove EDTA. The pooled gradient fractions were tested for the presence of calmodulin by calmodulin radioimmunoassay and by their ability to stimulate calmodulin-depleted phosphodiesterase. The purity of the pooled fractions was checked by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on slab gels in Tris/glycine buffer, pH 8.3, essentially as described by Laemmli (1970). Samples were electrophoresed at 25 mA on a 15 cm 12.5% polyacrylamide slab gel containing 0.1% sodium dodecyl sulphate. The gel was stained with 0.2% Coomassie Brilliant Blue in 50% (w/v) trichloroacetic acid for 15 min at room temperature and subsequently destained with 7.5% (v/v) acetic acid/5% (v/v) methanol.

Chemical modification of arginine residues

Arginine residues were modified by treatment of the proteins with 50 mM-cyclohexane-1,2-dione in 0.15 M-sodium borate buffer, pH 9, for 3 h at 37°C, essentially by the method of Demaille *et al.* (1977). Control samples were treated in the same way in the absence of cyclohexane-1,2-dione. Since Tris buffers were shown to decrease the stability of the modified arginine product (Patthy & Smith, 1975), assays of (Ca²⁺ + Mg²⁺)-ATPase activity were performed in 55 mM-sodium maleate buffer, pH 6.9, before and after modification of the arginine residues.

Solubilization of (Ca²⁺ + Mg²⁺)-ATPase from human erythrocyte membranes

(Ca²⁺ + Mg²⁺)-ATPase was solubilized from erythrocyte membranes as described by Carafoli *et al.* (1980). Essentially, the membrane (approx. 4–5 mg of protein/ml) was treated with Triton X-100 (1 mg of Triton/mg of membrane protein) at 4°C for 10 min, followed by centrifugation at 100 000 *g* for 30 min. The supernatant, containing the enzyme activity, was carefully removed. Approx. 10–12% of the membrane protein was solubilized by this procedure.

Determination of protein

The protein contents of PKI, membranes or Triton X-100-solubilized preparations were determined by a modified method of Lowry *et al.* (1951). The protein was precipitated free of interfering substances with 1.5% (w/v) sodium deoxycholate and 8% (w/v) trichloroacetic acid (final concentrations) before its assay (Bensadoun & Weinstein, 1976). Bovine serum albumin was used as the standard protein.

Results

Bovine heart PKI obtained from Sigma and purified on a Whatman DE-52 DEAE-cellulose column was shown to be free of calmodulin by a number of criteria (Table 1). Pure PKI did not stimulate calmodulin-deficient phosphodiesterase, whereas the PKI preparation from Sigma before purification and pure calmodulin stimulated this enzyme. The absence of significant amounts of calmodulin from the purified PKI was confirmed by calmodulin radioimmunoassay [1.3 ng of calmodulin was found per 2 µg of PKI (Table 1), which is well below the threshold (50 ng) that we found would stimulate (Ca²⁺ + Mg²⁺)-ATPase activity]. Moreover, 0.1%-sodium dodecyl sulphate/12.5% polyacrylamide-gel electrophoresis showed a single protein band of M_r 14 000 (Fig. 1). There was no evidence of contaminating calmodulin or an impurity reported in PKI from skeletal muscle (S.

Table 1. *Determination of calmodulin in various protein preparations*

Calmodulin was determined by using the phosphodiesterase assay and calmodulin radioimmunoassay, as described in the text. The phosphodiesterase activity was 100 nmol of P/min per mg of protein in the absence of added proteins. Bovine heart and rabbit skeletal-muscle PKI were added at a concentration of 2 µg/0.9 ml in the phosphodiesterase assay, and calmodulin was added at 0.4 µg/0.9 ml.

Preparation	Phosphodiesterase activation (%)	Calmodulin by radioimmunoassay
Bovine heart PKI (Sigma) (2 µg)	75	75 ng/2 µg of protein
Rabbit skeletal-muscle PKI (Sigma) (2 µg)	None	0.16 ng/2 µg of protein
Purified bovine heart PKI (2 µg)	None	1.33 ng/2 µg of protein
Calmodulin (400 ng)	230	420 ng

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of PKI and calmodulin

The proteins were separated on a 0.1%-sodium dodecyl sulphate/12.5%-polyacrylamide slab gel as described in the Experimental section. The following proteins were loaded on the gel (starting from left): lane 1, standard-molecular-weight proteins, 3 µg each of phosphorylase b (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soya-bean trypsin inhibitor (M_r 21 000) and lysozyme (M_r 12 300); lane 2, 10 µg of calmodulin; lane 3, 2 µg of rabbit skeletal-muscle PKI contaminant; lane 4, 10 µg of PKI (bovine heart); lane 5, 5 µg of PKI (bovine heart); lane 6, 2 µg of calmodulin.

Whitehouse & D. A. Walsh, personal communication).

Pure PKI from bovine heart stimulated ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase activity both in erythrocyte 'ghosts' and in Triton X-100-solubilized preparations (Fig. 2). However, whereas calmodulin stimulated ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase activity at both low (0.58 µM) and high (55 µM) concentrations of free Ca^{2+} , bovine heart PKI stimulated only at the low Ca^{2+} concentration, both in erythrocyte 'ghosts' (Fig. 2) and in Triton X-100-solubilized preparations (results not shown). At 0.58 µM- Ca^{2+} bovine heart PKI stimulated ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase in the presence of a saturating concentration (3 µg/0.6 ml) of calmodulin, whereas at 55 µM- Ca^{2+} PKI did not further stimulate above that in the presence of calmodulin.

The concentration-dependence of activation of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase by bovine heart PKI and calmodulin were investigated at 0.58 µM- Ca^{2+} (Fig. 3a). Although the enzyme activity was activated maximally at a lower concentration of PKI (2 µg/0.6 ml) than of calmodulin (3 µg/0.6 ml), the maximum activation by calmodulin was higher than that of PKI. Next we investigated the effect of PKI on the kinetics of Ca^{2+} activation of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase (Fig. 3b). In the absence of the activators the Ca^{2+} activation was biphasic, which we have previously ascribed to a mixed population of high- Ca^{2+} -affinity and low- Ca^{2+} -affinity states of the enzyme (Al-Jobore *et al.*, 1981; Al-Jobore & Roufogalis, 1981). The K_d of the high- Ca^{2+} -affinity component is approx. 2 µM and the K_d for the low- Ca^{2+} -affinity component is 40 µM or more. In the presence of either calmodulin or PKI, the low- Ca^{2+} -affinity component was virtually abolished, and the activation was fitted by a single high- Ca^{2+} -affinity state (K_d 0.4 µM) (Fig. 3b). However, unlike calmodulin, PKI did not increase the $V_{\max}$ of the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase activity. The shift in the Ca^{2+} -sensitivity of the enzyme by PKI and calmodulin and the increase in $V_{\max}$ by calmodulin are seen more clearly in Eadie-Hofstee plots of the data (Fig. 3c).

Skeletal-muscle PKI either from Sigma or purified to homogeneity (gift from S. Whitehouse & D. A. Walsh, University of California, Davis, CA, U.S.A.) failed to stimulate ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase in erythrocyte-'ghost' membranes at either low or high Ca^{2+} concentrations (results not shown). Moreover, an isolated contaminant of the PKI preparation from rabbit skeletal muscle, with physicochemical properties similar to those of PKI and calmodulin (S. Whitehouse, personal communication) (see Fig. 1), was found not to stimulate the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase activity (results not shown). In contrast with the differences observed in the stimulation of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase, PKI from skeletal muscle

Fig. 2. Effects of calmodulin and of bovine heart PKI on (Ca²⁺ + Mg²⁺)-ATPase activity in erythrocyte membranes and Triton X-100-solubilized enzyme at 0.58 μM- and 55 μM-free Ca²⁺

(Ca²⁺ + Mg²⁺)-ATPase activity in erythrocyte 'ghost' membranes (DG) was measured at 2 mM-ATP for 1 h at 37°C and solubilized enzyme activity (SE) at 0.5 mM-ATP for 10 min (for details of incubation mixture see the Experimental section). A, PKI (2 μg/0.6 ml); B, calmodulin (3 μg/0.6 ml); C, PKI (2 μg/0.6 ml) + calmodulin (3 μg/0.6 ml). Error bars represent the standard error for four experiments. Analysis of variance showed that all differences were significant at 0.58 μM-Ca²⁺ for DG and SE, whereas at 55 μM-Ca²⁺ DG and A were significantly different from B and C.

and bovine heart were equally effective in inhibiting cyclic AMP-dependent protein kinase (results not shown).

Evidence was obtained that the activation of (Ca²⁺ + Mg²⁺)-ATPase by PKI was not due to inhibition of endogenous cyclic AMP-dependent protein kinase. Cyclic AMP (5 μM) produced a small (approx. 20%) inhibition of (Ca²⁺ + Mg²⁺)-ATPase activity in erythrocyte 'ghosts' assayed at 20 μM-ATP, probably by phosphorylation of cyclic AMP-dependent protein kinase, since this effect was blocked by skeletal-muscle PKI (Fig. 4). Bovine heart PKI stimulated (Ca²⁺ + Mg²⁺)-ATPase activity by about 100% in the presence or in the absence of cyclic AMP (Fig. 4). Whereas cyclic AMP had no effect at 2 mM-ATP (results not shown), bovine heart PKI stimulated (Ca²⁺ + Mg²⁺)-ATPase at this ATP concentration, both in the absence (Figs. 2 and 3) and in the presence (results not shown) of cyclic AMP.

Bovine heart PKI also stimulated (Ca²⁺ + Mg²⁺)-ATPase in erythrocyte 'ghosts' treated with EDTA (EDTA-treated membranes), which are depleted of endogenous calmodulin (Roufogalis & Mauldin, 1980) (results not shown). These membranes also lack the endogenous cyclic AMP-dependent protein

kinase (Boivin & Galand, 1977), which further suggests that bovine heart PKI did not stimulate (Ca²⁺ + Mg²⁺)-ATPase by an action on cyclic AMP-dependent protein kinase.

Demaille *et al.* (1979) showed that modification of the arginine residues of PKI abolished its inhibition of cyclic AMP-dependent protein kinase. Modification of arginine residues of bovine heart PKI or of calmodulin had no effect on their ability to stimulate (Ca²⁺ + Mg²⁺)-ATPase activity (Table 2). However, modification of the arginine residues of PKI antagonized its inhibition of the phosphorylation of histone by cyclic AMP-dependent protein kinase.

Trifluoperazine has been shown to inhibit calmodulin stimulation of (Ca²⁺ + Mg²⁺)-ATPase (Levin & Weiss, 1979). Trifluoperazine (20 μM) inhibited activation of (Ca²⁺ + Mg²⁺)-ATPase by PKI calmodulin (Table 3). By contrast, the ability of PKI to inhibit cyclic AMP-dependent protein kinase was not affected by trifluoperazine (Table 4), suggesting that different sites or conformations of the PKI molecule may be involved in stimulation of (Ca²⁺ + Mg²⁺)-ATPase and in inhibition of cyclic AMP-dependent protein kinase (unless trifluoperazine interacts with the activated state of the ATPase rather than with PKI). It is noteworthy also that

Fig. 3. Activation of $(Ca^{2+} + Mg^{2+})$ -ATPase in human erythrocyte membranes by calmodulin and by bovine heart PKI (a) $(Ca^{2+} + Mg^{2+})$ -ATPase activity was determined at $0.58 \mu\text{M}$ Ca^{2+} , with PKI ($\blacktriangle$) and calmodulin ($\circ$). (b) Ca^{2+} activation of $(Ca^{2+} + Mg^{2+})$ -ATPase in human erythrocyte membranes was determined in the absence ($\blacktriangle$) and in the presence of 3 μg of calmodulin/0.6 ml ($\square$) and 2 μg of bovine heart PKI/0.6 ml ($\circ$). (c) Eadie-Hofstee plot of the data in (b), in the absence ($\blacktriangle$) and in the presence of 3 μg of calmodulin/0.6 ml ($\square$) and 2 μg of bovine heart PKI/0.6 ml ($\circ$). For experimental details see the Experimental section.

inhibition of cyclic AMP-dependent protein kinase by PKI does not require Ca^{2+} , as inhibition of histone phosphorylation occurs even in the presence of EGTA (results not shown).

Discussion

The interdependency of various cyclic AMP-dependent and Ca^{2+} -dependent cellular events is not clearly understood. Calmodulin has been shown to

stimulate brain adenylate cyclase, phosphodiesterase and $(Ca^{2+} + Mg^{2+})$ -ATPase enzymes, among others (Cheung, 1980). The PKI species have been shown to inhibit phosphorylation by cyclic AMP-dependent protein kinases by interacting with the free catalytic subunits of these enzymes (Ashby & Walsh, 1973). PKI also inhibits calmodulin-stimulated phosphodiesterase from Sertoli cells of testes, which contain both calmodulin and PKI (Beale *et al.*, 1977). In the present study we have shown that

Fig. 4. Effects of cyclic AMP and of PKI on (Ca²⁺ + Mg²⁺)-ATPase activity in human erythrocyte membranes at various concentrations of free Ca²⁺

(Ca²⁺ + Mg²⁺)-ATPase was determined in erythrocyte-ghost membranes in the absence (▲) and in the presence of 5 μM-cyclic AMP (○), 5 μM-cyclic AMP plus 2 μg of rabbit skeletal-muscle PKI/0.6 ml (▼), 2 μg of bovine heart PKI/0.6 ml (□) and 5 μM-cyclic AMP plus 2 μg of bovine heart PKI/0.6 ml (Δ). (Ca²⁺ + Mg²⁺)-ATPase activity was measured at 20 μM-ATP for 3 min. The rest of the experimental procedure was the same as that described in the Experimental section.

purified PKI from bovine heart stimulated the (Ca²⁺ + Mg²⁺)-ATPase of the human erythrocyte to an activity above that expected if it inhibited cyclic AMP-dependent protein kinase. The absence of calmodulin from the purified PKI was confirmed by two criteria: (a) the inability of purified PKI to stimulate calmodulin-deficient phosphodiesterase and (b) by calmodulin radioimmunoassay. In addition, no calmodulin was detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Though the gels do not show evidence of the presence of a calmodulin-like protein of similar mobility to bovine heart PKI, this possibility cannot be eliminated entirely. The PKI from rabbit skeletal muscle and a related anionic protein from the rabbit skeletal muscle did not stimulate the (Ca²⁺ + Mg²⁺)-ATPase, even though their isoelectric points are similar to that of the bovine heart PKI (Whitehouse *et al.*, 1980). The reason for this is not known, but it may be due to species differences in the amino acid composition of the proteins. Whitehouse *et al.* (1980) have demonstrated three charge isomers of PKI from bovine heart, all of which were capable of inhibiting the cyclic AMP-dependent protein kinase. Because all the charge isomers were presumably present in the present study, it is not known if any specific charge isomer is more potent than the others in stimulating (Ca²⁺ + Mg²⁺)-ATPase.

The PKI stimulation of (Ca²⁺ + Mg²⁺)-ATPase

Table 2. Effect of modification of arginine residues of bovine heart PKI and of calmodulin on modulation of (Ca²⁺ + Mg²⁺)-ATPase activity or cyclic AMP-dependent protein kinase catalytic subunit (C-subunit) activity

Modification of arginine residues of PKI and of calmodulin and the assay of histone phosphorylation and ATPase activity were as described in the Experimental section.

Reagent	Residue modified	C-subunit-catalysed transfer of ³² P _i to histone (pmol of P _i transferred/10 min)	(Ca ²⁺ + Mg ²⁺)-ATPase activity (μmol of P _i ml of 'ghosts')	
			PKI	Calmodulin
Unmodified	—	38 (140*)	3.5 (1.8*)	4.5 (1.8*)
Cyclohexane-1,2-dione	Arginine	130	3.5	4.3

* The value in parenthesis represents the control value without PKI or calmodulin.

Table 3. Effect of trifluoperazine on the ability of bovine heart PKI to stimulate (Ca²⁺ + Mg²⁺)-ATPase in erythrocyte 'ghost' membranes

When used, trifluoperazine was added to the reaction mixture in the dark 10 min before the reaction was started with ATP. (Ca²⁺ + Mg²⁺)-ATPase activity was assayed at 2 mM-ATP and 0.58 μM-Ca²⁺, as described in the Experimental section.

Enzyme preparation	Trifluoperazine (20 μM)	(Ca ²⁺ + Mg ²⁺)-ATPase (μmol of P _i /h per ml of 'ghosts')
Erythrocyte 'ghosts'	—	1.5
	+	1.4
Erythrocyte 'ghosts' + PKI (2 μg)	—	3.2
	+	1.6
Erythrocyte 'ghosts' + calmodulin (3 μg)	—	4.0
	+	1.75

Table 4. *Effect of trifluoperazine on the inhibition of cyclic AMP-dependent protein kinase catalytic-subunit-catalysed phosphorylation of histone by bovine heart PKI*

Histone phosphorylation was performed as described in the Experimental section. Trifluoperazine and PKI were preincubated with the reaction mixture for 10 min in the dark before the reaction with ATP was started.

Assay conditions	Histone phosphorylation (pmol of $^{32}\text{P}_i$ transferred/10 min)
Control	140
PKI (2 μg)	38
Trifluoperazine (20 μM)	140
PKI (2 μg) + trifluoperazine (20 μM)	40

resembles that of calmodulin, as both increase the sensitivity for Ca^{2+} . However, unlike calmodulin, PKI does not increase the maximum velocity. The stimulation by PKI at low Ca^{2+} concentrations is not due solely to an effect on the phosphorylation of cyclic AMP-dependent protein kinase, for several reasons, as follows. (a) Cyclic AMP caused a comparatively small inhibition of the enzyme activity (approx. 20%) at all Ca^{2+} concentrations used, whereas the stimulation by PKI is about 100% and independent of added cyclic AMP. Furthermore, PKI stimulation occurred at 2 mM-ATP, conditions under which cyclic AMP inhibition of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase was not observed. (b) Modification of arginine residues of PKI led to a loss in the inhibition by PKI of histone phosphorylation by cyclic AMP-dependent protein kinase, but had no effect on the stimulation of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase. This suggests that the sites of interaction of PKI with $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase and with protein kinase are different or that arginine residues are not required for activation of ATPase, as is also the case with calmodulin (Table 2). (c) PKI stimulated EDTA-treated membranes, which are depleted of cyclic AMP-dependent protein kinase (Boivin & Galand, 1977).

Khandelwal *et al.* (1980) have reported that under certain conditions the 'phosphoprotein phosphatase' activity of liver is increased by calmodulin and by troponin C, and they suggested that this effect may be due to the negative charge of these compounds, as the effect showed lack of Ca^{2+} -dependency. Although it is possible that the increase in $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity by PKI observed in the present study could be due to increased phosphatase activity, as a result of its negative charge alone, this is less likely because rabbit skeletal-muscle PKI, which has a similar charge, was ineffective and the effect is observed in EDTA-treated membranes, which lack the cyclic AMP

phosphorylating system. The inhibitory effect of trifluoperazine on both PKI and calmodulin stimulation of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase suggests that caution should be used in invoking a role for calmodulin in a cellular process on the basis of this evidence alone. The lack of trifluoperazine effect on phosphorylation of cyclic AMP-dependent protein kinase in the presence (or in the absence) of PKI further supports the conclusion that different mechanisms or interactions are involved in the two actions of PKI.

In summary, we have shown that cyclic AMP produced an inhibition of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity, probably via cyclic AMP-dependent protein kinase-mediated phosphorylation, since this effect was abolished by PKI from rabbit skeletal muscle. PKI from bovine heart stimulates $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase at low Ca^{2+} concentrations in the presence and in the absence of cyclic AMP, by increasing the enzyme's sensitivity for free Ca^{2+} without affecting its maximum velocity. The stimulation of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase at low Ca^{2+} concentrations by PKI is seen even in the presence of saturating concentrations of calmodulin. The partial reactions of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase-catalysed reaction sequence affected by this class of activators remain to be established.

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